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Studies on Salt Fish. VIII. Effects of Various Salts on Preservation

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(Received for publication January 23, 1942)

ABSTRACT

Spoilage, as measured by rise in trimethylamine and bacterial content, of cod press-juice containing 21.0 g. salt per 100 ml. (approximately 80 per cent saturated) is delayed longest with pure sodium chloride, and increasingly less with mined evaporated, mined crude, Mediterranean and Turk's Island solar salts. This order corresponds to decreasing percentages of sodium chloride and increasing percentages of impurities in these salts. The differences in the salt action increase with lower temperatures.

INTRODUCTION

In order to ascertain the relative effect of commercial fishery salts as compared with pure sodium chloride upon the preservation of cod press-juice, a series of experiments were carried out, using selections of the following salts: Sodium chloride, c.p.; Liverpool, mined, evaporated; Malagash, mined, evaporated; Malagash Fisheries, mined, crushed; Mediterranean, solar; Turk's Island, solar.

It is a well known fact that neither the mined nor the solar salts have a uniform composition, but that each batch varies in the amount of impurities such as calcium chloride, calcium sulphate, magnesium chloride, magnesium sulphate, it contains.

TABLE I. Average composition of various mined and solar salts, as calculated from amounts of sodium, calcium, magnesium, chloride and sulphate determined by analysis. From data of Wilson (1938) and on our files (unpublished).

No. of analyses	Salt	NaCl	CaCl ₂	CaSO ₄	MgCl ₂	MgSO ₄	Insoluble
5	Liverpool.....	96.7-99.96	0-0.113	0.675-1.26	0-0.08	0-0.04	0.01-0.035
	Average....	98.00	0.033	0.769	0.041	0.011	0.023
4	Malagash Evaporated..	95.83-98.3	0-0.02	0.352-1.401	0.002-0.082	0-0.014	0.003-0.143
	Average....	97.027	0.005	0.895	0.028	0.005	0.045
12	Malagash Fisheries....	93.8-98.3	0-0.42	0.071-1.81	0.005-1.368	0-0.701	0.560-1.56
	Average....	96.86	0.075	1.409	0.253	0.094	0.992
18	Mediterranean*.....	93.15-99.5	0-0.43	0.03-3.97	0-1.109	0-1.63	0.005-0.89
	Average....	96.985	0.085	0.679	0.258	0.302	0.140
9	Turk's Island.....	94.2-99.09	0-0.723	0.332-3.39	0.01-1.562	0-1.08	0.05-1.037
	Average....	96.027	0.121	1.839	0.613	0.270	0.384

*Including Cadiz, Cagliari, Torreveija, Trapani, Sfax, Port Said.

It can be seen in table I that the Liverpool salts are on the average highest in sodium chloride content, the Malagash and Mediterranean salts intermediate, and the Turk's Island salts lowest. At the same time, with two notable exceptions (Mediterranean salts relatively low in calcium sulphate and Malagash Fisheries salt relatively high in insoluble matter), the various impurities are found to occur in increasing amounts in the order: mined evaporated salts, mined crude salts, Mediterranean salts and Turk's Island salts.

It has been shown by Tressler (1920) that these impurities have a definite impeding action on the permeability of the cell membranes, and consequently slow down salt penetration into the fish muscle tissues, thereby indirectly affecting preservation. The use of fish muscle press-juice instead of fish muscle itself in the present investigations not only makes bacterial counts more reliable but also eliminates the salt penetration factor. The question naturally arises: will commercial salts of such varying composition differ essentially in their effects on bacteria and thus directly affect preservation? At least two possibilities present themselves: that the major impurities, such as those listed in table I, are responsible for any variation, if such should be found, or that trace elements that are not usually estimated in the routine analysis of commercial salts may be the determining factors in any difference in the effects of these salts. The first possibility has been investigated.

METHODS

The technique used is essentially that employed by Labrie and Gibbons (1937). Press-juice was obtained from pre-rigor cod muscle and made up with the salt, which had been previously dried in the vacuum oven at 105°C., in volumetric flasks in the proportion of 210 g. salt dissolved in some 950 ml. juice and made up to one litre, resulting in an approximately 80 per cent saturated solution in the case of the pure sodium chloride. The resulting culture medium with its chance inoculation of fish slime bacteria was incubated in flasks or tubes at constant temperatures.

This salt concentration was chosen as approaching the fully saturated conditions of actual practice without unduly prolonging the length of the experiments. Labrie and Gibbons (1937) found that at this salt concentration of the press juice, which corresponds to 26.5 g. of salt added to 100 ml. of press-juice (between their so-called 24 and 28 per cent media), the bacterial counts on 10 per cent and 20 per cent salt agar were practically identical.

Bacterial counts were made on salt agar, using saline dilution flasks, each containing 9.1 g. sodium chloride per 100 ml. Trimethylamine production was followed by the Beatty-Gibbons (1936) and Watson (1939) adaptations of the Conway and Byrne (1933) test.

In this paper the salt concentration of press-juice when expressed as percentage denotes the number of grams of salt per 100 ml. of juice.

EXPERIMENTAL WORK

AEROBIC AND ANAEROBIC CONDITIONS

Watson (1939) noticed that trimethylamine production in fresh cod press-juice proceeded less rapidly for the first four days of incubation at 2°C. under

aerobic conditions—using a thin layer of culture medium in a large Erlenmeyer culture flask and keeping it aerated—than under anaerobic conditions with the culture in stoppered flasks or deep tubes filled completely with the culture medium.

This observation was checked with salted press-juice, using both chemically pure sodium chloride and Turk's Island salt, at 20°C. In these experiments the opposite results were obtained, i.e. a prolonged lag occurred in the trimethylamine production when measured in deep tube cultures as compared with thin layer cultures in flasks. This may be at least partly due to differences in the bacterial populations, although Sigurdsson (unpublished data), using pure strains of bacteria capable of reducing trimethylamine oxide in a medium containing yeast extract, peptone, glucose, trimethylamine oxide, and salt in 0 to 6.5 per cent concentrations, found also that under anaerobic conditions bacterial growth and trimethylamine production were slower than under aerobic culture conditions. In the present test it was also observed that in the press-juice containing Turk's Island salt more rapid trimethylamine production took place than in the pure sodium chloride press-juice in both cases. At the same time it was noticed that the differences in the effects of the two salts were relatively smaller than the differences due to the method of culture—aerobic versus anaerobic conditions—, so that the trimethylamine production was actually more rapid in the sodium chloride *flask* cultures than in the Turk's Island salt *deep tube* cultures. The time period necessary to reach a trimethylamine level of 4.0 mg. per 100 ml.—the odour threshold value for fresh press-juice—is as follows in days in each case: Turk's Island salt, flask culture, $19\frac{1}{2}$; sodium chloride, flask culture, $23\frac{1}{4}$; Turk's Island salt, deep tube, $26\frac{1}{2}$; sodium chloride, deep tube, $31\frac{1}{2}$.

REACTION AND BUFFERING

In general in the preparation of cultures of the salted press-juice with various salts, the initial hydrogen-ion concentration showed less variation between the different salts used in any one test, than between test runs using different press-juice. For example, in one test the sodium chloride press-juice had an initial pH value of 6.03 and the Turk's Island one of 6.08, while in another test the values were 6.60 and 6.75, respectively.

Salted press-juice was prepared as previously described, using sodium chloride in one case and Turk's Island salt in the other. Each lot was divided into three portions: the first was left unbuffered (initial hydrogen concentration, pH 6.05); the second was buffered with KH_2PO_4 and NaOH to pH 5.64; and the third was buffered to pH 8.0, as measured by means of the Beckman glass electrode. The six cultures were incubated in Erlenmeyer culture flasks at 25°C. and the changes in trimethylamine, in counts of viable aerobic bacteria and in hydrogen-ion concentration were followed.

In each case the Turk's Island press-juice showed rapid trimethylamine production which preceded that in the corresponding sodium chloride press-juice.

In the unbuffered cultures, rapid initial bacterial growth occurred. This was followed, in the Turk's Island salt culture, by rapid trimethylamine production, causing a rapid change of the hydrogen-ion concentration which reached a

pH value of 7.8 in 16 days. In the sodium chloride culture, trimethylamine production with its accompanying rise in pH did not become rapid until after 18 days' incubation. Sigurdsson (unpublished data) also noticed a change in the reaction of an unbuffered medium towards the alkaline side during the course of trimethylamine production at low salt concentrations (0 to 6.5 per cent). He found that buffering at pH 7.0 caused an increase in growth and trimethylamine production.

As Watson (1939) showed, muscle press-juice is in itself a highly buffered medium, as compared with unbuffered laboratory media.

In the cultures buffered at pH 8.80, a gradual increase of the hydrogen-ion concentration occurred, reaching pH 8.38 after 30 days' incubation, i.e. practically the same final level as in the unbuffered cultures. The early bacterial increase was slower in these buffered cultures than in those unbuffered, but reached approximately the same maximum. Trimethylamine production was also slower in the buffered cultures, at least in the case of the Turk's Island salt culture.

In the acid buffered cultures, trimethylamine production was inhibited for a long period until the buffering capacity of the culture was overcome, after which a rapid increase occurred, and consequently the hydrogen-ion concentration was reduced. Total bacterial counts on 10 per cent salt agar showed comparatively rapid increase in numbers, and reached the highest total. Obviously organisms that reduce trimethylamine oxide were not favoured by the initial low pH value of the culture. This confirms Nadeau's (1940) finding on fresh fillets: that buffering at approximately pH 5.0 has an inhibiting effect on trimethylamine production.

The time periods (in days) to reach a trimethylamine level of 4.0 mg. per 100 ml. of press-juice were as follows:—Turk's Island culture, unbuffered and buffered at pH 8.8, 12; sodium chloride culture, buffered at pH 8.80, 13; sodium chloride culture, unbuffered, 14½; Turk's Island culture, buffered at pH 5.64, 19; sodium chloride culture, buffered at pH 5.64, 26.

TYPE OF SALT

All salts were dried in the vacuum oven at 105°C. Three tests were carried out: the first with sodium chloride c. p. and Turk's Island salt (solar) at 25°C.; the second with sodium chloride c. p., Malagash fishery salt (mined) and Turk's Island salt at 21°C.; and the third with sodium chloride c. p., Liverpool evaporated salt, Malagash evaporated salt, Malagash fishery salt, Mediterranean salt (solar) and Turk's Island salt at 15.5°C.

The trimethylamine curves (fig. 1) are similar in shape, but show an increasingly longer lag period advancing from solar to mined to pure salt. The 4.0 mg. trimethylamine level was reached after 19½ days with the solar salt, after 24½ days with the mined salt, and only after 26 days with the pure sodium chloride.

These findings, indicating that solar salt in high concentration is less growth-retarding than mined salt, and both of them less so than pure sodium chloride, fit in very well with observations made by Sigurdsson (unpublished data) that at those low salt concentrations (2 to 4 per cent) where bacterial growth and trime-

thylamine production are actually stimulated, the pure sodium chloride has the least stimulating effect and the solar salt the most, and that at the higher concentrations investigated (5.7 to 7.4 per cent) pure sodium chloride is more inhibitory than mined or solar salts.

At 25°C., the lag for the solar salt was also shorter than that for the pure salt, and their lengths (10½ and 14 days respectively) were less than at the lower

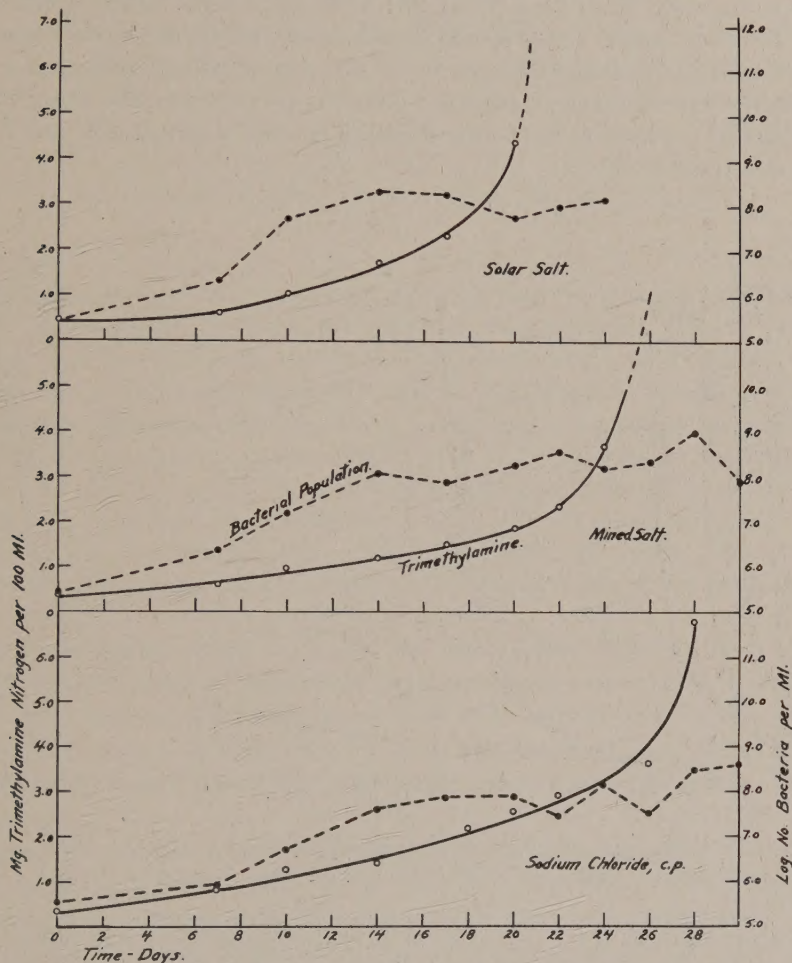


FIGURE 1. Trimethylamine production and increase in bacterial population in cod press-juice containing 21.0 g. solar salt, mined salt, and sodium chloride, c.p. per 100 ml. respectively, at 21°C.

temperature. At 15.5°C. the relative lengths of all lag periods were increased considerably, but they remained in the same order, the 4.0 mg. trimethylamine level being reached in 52½ days with the Turk's Island salt, in 65 days with the Mediterranean salt, in 74 days with the Malagash fishery salt, in 87 days with the Malagash evaporated salt, in 95 days with the Liverpool salt and not until 109½ days with the pure sodium chloride.

In figure 2 the effects of temperature as shown in these series are summarized. They not only confirm Labrie and Gibbons' (1937) finding for sodium chloride of a prolonged lag period with lower temperatures of incubation, but show that the spread between the sodium chloride and the solar salt curves becomes increasingly wider with a decrease in the incubation temperature. At the 4.0 mg. trimethylamine level, the time interval between the sodium chloride and solar salt curves increased from $3\frac{1}{2}$ days at 25° to $6\frac{1}{2}$ days at 21° and to 57 days at 15.5°C . In other words, at the lower temperatures, which are more desirable in commercial salting procedure, the purity of the salt is of increasing importance, i.e. the relative effectiveness of pure sodium chloride over solar salt in inhibiting trimethylamine production is 16 times higher at 15.5° than at 25° and 9 times higher than it is at 21°C .

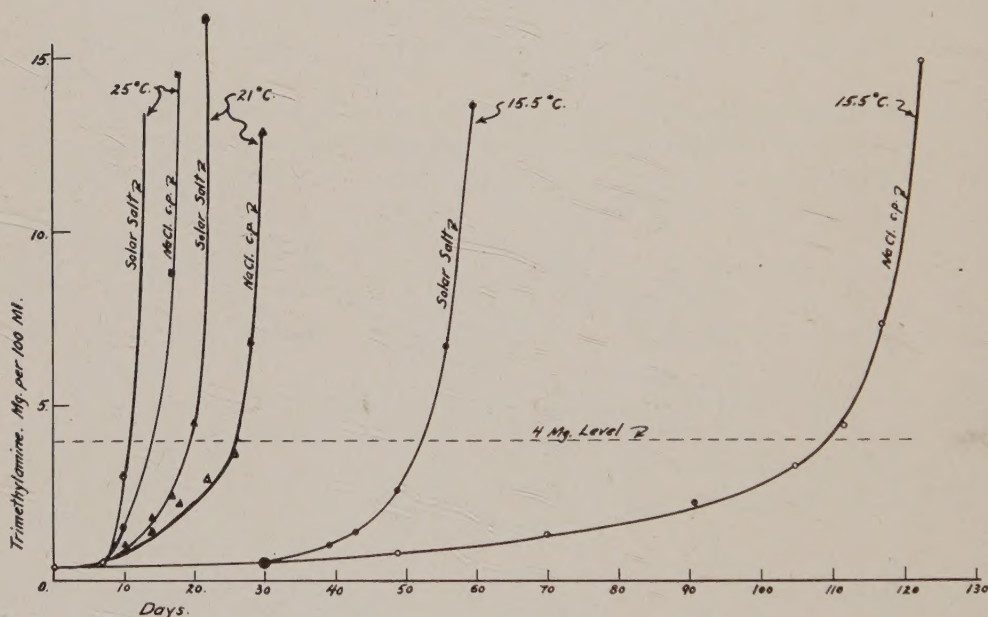


FIGURE 2. Effect of temperature on trimethylamine production in cod press-juice containing 21.0 g. solar salt and 21.0 g. sodium chloride c.p. per 100 ml., respectively.

As Labrie and Gibbons (1937) found for salt concentration and temperature, we find that the types of salt and the temperature affect the length of the initial lag period in the production of trimethylamine, and that, once this production becomes noticeable, it will proceed at a very rapid rate, practically independent of salt concentration, temperature and type of salt. Beatty and Gibbons (1936) observed similar conditions concerning the effect of temperature upon trimethylamine production in fresh cod press-juice.

Counts of viable bacteria gave practically the same result in all cases. After an initial lag period, the length of which depended primarily on temperature, and was approximately 4 days at 25° , 7 days at 21° and 35 to 40 days at 15.5°C ., a rapid increase in the bacterial population occurred, which always preceded any

significant rise in trimethylamine production. Labrie and Gibbons (1937) also found that no trimethylamine formation was noticeable until the bacterial population had reached a certain level, approximately 10 to 20 millions per ml. During this initial period of bacterial growth, the organisms use the oxygen in their immediate environment as a hydrogen acceptor until the oxidation-reduction potential reaches the level at which trimethylamine oxide reduction sets in, as demonstrated by Watson (1939).

In our tests it was noticed, in practically all cases, that after an initial peak in the population had been reached, a stationary period or even a slight decrease in number of viable bacteria occurred. Qualitative examination of the plates showed that during this period a shift in the population usually occurs, accounting for the second increase in bacterial number. This last phase coincides as a rule with the period of rapid trimethylamine production (figure 1).

IMPURITIES

Shaughnessy and Winslow (1927) showed that for *Es. coli* calcium chloride even in high dilutions tends to decrease the permeability of the cell walls, thus affecting viability and biochemical activity unfavourably. Fillon (1924) observed the same effects of calcium chloride, magnesium chloride and magnesium sulphate in presence of high sodium chloride concentrations upon red halophilic bacteria. Boury (1934) also noticed similar effects of calcium chloride and sulphate on red halophiles. Small amounts of magnesium salts (chloride and sulphate), on the other hand, were found to stimulate their growth in presence of high concentrations of pure sodium chloride. The magnesium salts when present together with small amounts of calcium chloride were found to annul the inhibitory effect of the latter.

Gibbons (unpublished data), on the other hand, observed that a medium containing no calcium or magnesium ions (prepared from dialyzed drip of fresh fish muscle, washed agar and pure sodium chloride) did not support growth of red halophilic bacteria or only very poorly, whereas addition of traces of calcium or magnesium or replacement of the sodium chloride by solar salt resulted in good growth.

Two series of press-juice and salt media were made up, using a basic concentration of 20.16 g. sodium chloride per 100 ml. of medium. This corresponds in sodium chloride content to the standard Turk's Island salt media employed in all our experiments, which contained 21.0 g. of the solar salt per 100 ml. of medium. Turk's Island salts contain an average of 96 per cent sodium chloride (table I). Addition to this basic medium of 0.84 g. sodium chloride per 100 ml. medium gave the standard sodium chloride press-juice containing 21.0 g. of this salt per 100 ml. In the first series this additional salt was replaced by magnesium sulphate or calcium chloride both on a weight and a molecular basis. All media were incubated at 23°C. and the increase in trimethylamine determined periodically. The results after 10 days' incubation are shown in table II, indicating that the effects of all salts added to the basic medium in the concentrations used are additive in their inhibitory action, particularly in case of the calcium chloride.

TABLE II. Trimethylamine after 10 days' incubation at 23°C. in cod press-juice with varied salt content.

No.	Medium	NaCl	Additional salt	Trimethylamine (mgm. per 100 ml.)
		(g. per 100 ml.)		
1	Basic.....	20.16		38.0
2	Standard NaCl.....	20.16	0.84 (NaCl) (0.144 m)	16.0
3	Basic + MgSO ₄	20.16	0.84 (MgSO ₄)	18.0
4	Basic + CaCl ₂	20.16	0.84 (CaCl ₂)	1.0
5	Basic + MgSO ₄	20.16	1.73 (MgSO ₄) (0.144 m)	3.0
6	Basic + CaCl ₂	20.16	1.60 (CaCl ₂) (0.144 m)	1.0

In the second series the basic medium containing 20.16 g. sodium chloride per 100 ml. and the standard sodium chloride medium (containing 20.16+0.84 g. =21.0 g.) were prepared. In addition a natural Turk's Island salt medium containing 21.0 g. solar salt per 100 ml., 96 per cent of which (20.16 g.) are sodium chloride, and 4 per cent (0.84 g.) are impurities, was used. A synthetic solar salt medium containing 20.16 g. pure sodium chloride, and pure calcium chloride, calcium sulphate, magnesium chloride and magnesium sulphate in the proportions indicated in table I for the average composition of Turk's Island salt, was prepared also. Further basic media to which each of the above four salts were added separately in the same amounts as above were used.

TABLE III. Trimethylamine after 8 days' incubation at 23°C. in cod press-juice with varied salt content.

No.	Medium	NaCl	Additional salt	Trimethylamine (mgm. per 100 ml.)
		(g per 100 ml.)		
1	Basic.....	20.16		40.0
2	Standard NaCl.....	20.16	0.84 (NaCl)	20.0
3	Standard T. Island.....	20.16	0.84 (impur.)	38.0
4	Synthetic T. Island.....	20.16	0.6 (other salts)	29.0
5	Basic+CaCl ₂	20.16	0.025 (CaCl ₂)	26.0
6	Basic+MgSO ₄	20.16	0.057 (MgSO ₄)	40.0
7	Basic+CaSO ₄	20.16	0.386 (CaSO ₄)	46.0
8	Basic+MgCl ₂	20.16	0.129 (MgCl ₂)	48.0

The results (table III) seem to indicate that the lesser retarding action of the solar salt in medium no. 3, as compared with the standard sodium chloride medium, each of which contains 21.0 g. of salt per 100 ml., is due to the lower amount of sodium chloride present in the solar salt. In fact, the results for the solar salt medium are practically identical with those for the basic medium which contains the same amount of sodium chloride, whereas in the standard sodium chloride medium the retarding effect is very great, as indicated by a 50 per cent reduction in trimethylamine production. The effects of the impurities in the

natural solar salts appear to neutralize each other. The salts in the synthetic solar salt had a slightly greater retarding effect than the ones in the natural solar salt, the effect being about halfway between that in the standard sodium chloride medium no. 2 and that in the standard solar salt medium no. 3 or the basic medium no. 1. Where the four impurities were added singly the magnesium sulphate had no effect, i.e. it had neither a stimulating nor a retarding effect, whereas the calcium chloride had a slightly retarding effect. The calcium sulphate and magnesium chloride salts on the other hand in the amounts present in solar salt were slightly stimulating, as compared with the basic medium. Winslow and Dolloff (1928) observed that while in dilute solutions, the favourable (stimulating) effects of two salts (one monovalent and the other bivalent) are additive, and in the higher concentrations the toxic effects are additive, in the middle range (where one salt is favourable and the other toxic) the mixture gives an intermediate effect.

It is realized that in the media used (cod press-juice) unknown and varying amounts of different cations are added to the system apart from the salts used in the media. Further investigations will therefore be based on work with laboratory media of known composition and with pure cultures of spoilage organisms.

SUMMARY

When cod press-juice is approximately 80 per cent saturated with salt (21.0 g. per 100 ml.), spoilage, as measured by rapid increase in trimethylamine and rise in bacterial population, occurs after lag periods of different length, depending upon the type of salt used, Turk's Island (solar) salt delays spoilage least, while the other salts cause increasing lag periods in the order named: Mediterranean solar salts, mined crude salts, mined evaporated salts, pure sodium chloride. The differences in the effects of the various salts are increasingly evident with a lowering of the incubation temperature.

Spoilage is delayed with sodium chloride and solar salts under anaerobic conditions and when the salted press-juice is buffered between pH 5 and 6.

The less severe effect of solar (Turk's Island) salt in high concentration appears to be due partly, at least, to its lower sodium chloride content (96 per cent), while the impurities appear to neutralize each other, the calcium chloride being slightly inhibitory and the magnesium chloride and calcium sulphate slightly stimulating in the presence of the high amounts of sodium chloride.

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Studies on Salt Fish.
IX. Effect of Environment upon Growth
of Red Halophilic Bacteria

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(Received for publication January 13, 1942)

ABSTRACT

Growth of 25 strains of red halophiles stopped below 15° and above 55°; heat resistance is less than two minutes at 98°C. in 24 per cent saline. Range of relative humidity necessary for growth lies between 70 and 85 per cent. They have high resistance to ultra-violet light, surviving 120 minutes' exposure. Range of salt tolerance can be increased in both directions by gradual adaptation. Replacement of the Na ion by other cations inhibits growth.

This investigation deals with the influence of environment, both physical and chemical, upon a number of such red halophiles as the thirty strains from reddened salt fish, salt and salt fish plants described by Gibbons (1937).

The basic medium used throughout was the modified skim-milk salt agar of Lochhead (1934) prepared by mixing equal amounts of re-heated sterile skim milk and liquefied sterile salt agar. The latter contained 450 parts sodium chloride and 15 parts agar in 1,000 parts of tap water. The skim milk was heated to 50°C. before mixing with the liquefied agar. The final medium contained 18.4 g. of sodium chloride in 100 ml., which salt concentration is expressed in this paper as 18.4 per cent.

A total of some two dozen red halophilic strains belonging to the genus *Serratia* and two *Sarcina* strains were used. About half the *Serratia* strains were strongly proteolytic.

TEMPERATURE RANGE

While it is well known that the optimum growth temperature of red halophilic bacteria is fairly high (around 40°C.), it is not generally realized that the temperature range is unusually wide. Gibbons (1937) obtained growth between 20° and 53°, Fillon (1924) observed growth at 10°, optimum at 42°, but none at 50°, Harrison and Kennedy (1922) found growth to occur between 10° and 46°, and Browne (1922) reports an optimum of 50° to 55°.

In the present tests, growth stopped for all (9) strains investigated between 10° and 15° and between 55° and 60°, using the skim-milk salt agar described above.

HEAT RESISTANCE

Harrison and Kennedy (1922) report resistance for 10 minutes to exposure at 62°C. but not at 65° for organisms in heavy suspension in a 16 per cent salt solution, and transferred to "16 per cent" salt culture media, i.e. containing 13.8 g. salt per 100 ml.

In the present tests near 100°, correspondingly lower heat resistance was encountered. At 99.4° the strain tested survived after two minutes' but not after three minutes' exposure in skim milk containing 13.5 per cent salt.

At 98.7° the same strain survived after three minutes' exposure in 27 per cent saline, but not after four minutes, and at 86° survival occurred after eight minutes' exposure but not after ten minutes; it survived exposure to 75° for 20 minutes and 60° for 32 minutes. Twenty-one strains exposed in a buffered 24 per cent saline (nearly saturated) did not survive two minutes' exposure to 98°.

Baumgartner (1937) has shown that for a halophilic organism the heat resistance at 60° decreased when the salt concentration of the buffer in which it was suspended was decreased.

RELATIVE HUMIDITY

While non-halophilic organisms require high relative humidities for growth, and Lloyd, Marriott and Robertson (1929) also claim 100 per cent relative humidity as optimum for red halophilic bacteria from salted hides, Frank and Hess (1941) have shown that for halophilic brown molds of the genus *Sporendonema* growth and spore formation are at an optimum at 75 per cent relative humidity.

A number of strains of halophilic *Serratia* were tested on a 22.5 per cent sodium chloride skim milk agar and at various temperatures. For this purpose, series of Spray dishes were set up containing each a relatively large amount (150 ml.) of suitably diluted sulphuric acid to obtain various relative humidities at different temperatures in the head space of the sealed dishes. One ml. each of sterile medium was allowed to solidify on sterile glass slides and these slides were inoculated with the test organisms and suspended in the head space of the Spray dishes. These were sealed immediately and incubated. This relative proportion of acid and medium assured a rapid equilibrium being established between the moisture in the head space of the dish and the medium without changing the former perceptibly. In table I the total ranges of relative humidity and the optima are given for the growth of various strains at different temperatures.

TABLE I. Range and optimum in relative humidity for growth of red halophilic bacteria.

Temperature (° C.)	55	40	37	30	20	15
Number of strains.....	9	9	19	9	9	9
Range (% rel. hum.).....	70-80	70-85	70-85	70-85	75-85	75-85
Optimum (% rel. hum.)....	75	75	75-80	75-80	80	80

It is obvious that, practically independent of temperature, the range is fairly constant, lying within 70 and 85 per cent relative humidity, the optimum falling

between 75 and 80 per cent. This agrees closely with the above-mentioned findings by Frank and Hess (1941) on halophilic molds.

The range corresponds to the one observed by Cooper (1938) for the equilibrium moisture coefficients of salt fish muscle. The media in our Spray dishes incubated at 70 per cent, and lower relative humidities became soon desiccated and did not allow growth, while in the media held at relative humidities above 85 per cent such an amount of moisture was adsorbed that the salt concentrations at the surface of the medium became too low for these halophilic organisms. Thus the relative humidity conditions which are necessary for the proper storage conditions of salt fish are unfortunately optimal also for the growth of these halophilic organisms responsible for the spoilage of a certain proportion of salt fish.

ULTRA-VIOLET LIGHT

Broadbent (1938) reported killing times of the order of about 25 to 75 seconds for such bacteria as *Es. coli*, *Staph. aureus* and *albus*, *B. subtilis* and yeasts, and 4 to 19 minutes for various molds upon exposure to ultra-violet light. Browne (1922) reported that red halophiles on solar salt will survive exposure to bright sunlight for eight hours and more. Frank and Hess (1941) found that exposure of halophilic brown molds (*Sporendonema epizoum*) to ultra-violet light (commercial type of Westinghouse "Sterilamp" 2537 Å units at a distance of 10 cm.) did not kill all cells or spores in 60 to 80 minutes.

Red halophilic bacteria were exposed to ultra-violet light from the same Sterilamp, either on the surface of skim-milk salt agar plates, on contaminated salt crystals or on contaminated slices of salt fish. Exposure up to 35 minutes failed to sterilize the salt or the fish. Plate cultures exposed for 60 minutes showed almost continuous growth over the plate after subsequent incubation, while, on similar plates exposed up to 120 minutes, growth occurred only in the form of a few separate large colonies, probably at the spots of heaviest inoculation.

It is obvious that these halophilic red bacteria, similarly to halophilic brown molds (*Sporendonema*), exhibit a much greater resistance toward ultra-violet light than has been reported for non-halophilic micro-organisms.

SALT TOLERANCE

It is generally considered that halophilic organisms arose from non-halophilic forms through adaptation to higher salt concentrations. Labrie and Gibbons (1937) and Hess (1942) have shown that fish press-juice obtained from pre-rigor muscle without aseptic precautions will spoil even if 24 to 28 g. of salt are added to 100 ml. of it. Hof (1935) isolated obligate halophiles from salt-free material (soil) and concluded that they were derived from ordinary soil bacteria. Stuart (1938) has shown that halophilic organisms can be isolated from sources of low salt content such as soil, water, and dung by cultivation for a prolonged period in an enrichment medium of high salt concentration. The British Salt Union (personal communication) reports that a large number of halophilic organisms have been isolated from seawater samples from many parts of the world. Harrison and Kennedy (1922) have isolated red halophiles from seawater in the presence of sterile Irish moss (*Chondrus crispus*) in the culture. Velu and Balozet (1929)

even claim that the red halophiles are sudden and irreversible mutations of *Bac. prodigiosus* (*Serratia marcescens*) and that there are no true halophiles. Stuart and James (1938) found that so-called halophilic organisms such as *Sarcina littoralis* show only obligate halophilic characteristics when transferred from young, rapidly growing cultures, but when allowed to age for 30 days or more they are capable of growth upon transfer to media of low salt content.

The question of the adaptation of the present twenty-five strains of red halophiles to lower or higher salt concentrations has been studied, using skim-milk agars containing sodium chloride from 3 per cent to 27 per cent (saturated) at 3 per cent intervals. From the stock cultures, growing at 18.4 per cent salt, all skim-milk salt agars were inoculated to determine the initial or original salt range of each culture. Then, after one month's incubation, in each series of sub-cultures those growing at the highest and lowest salt concentrations were transferred to the media containing 3 and 6 per cent more, or less salt, respectively. This was repeated following another month's incubation, when no further growth was observed after transfer to higher or lower salt concentrations. Table II shows the number of cultures growing over various salt ranges, first as originally tested, and second as tested after being subsequently transplanted twice at one-month intervals from the extremes to lower and higher salt concentrations.

It can be seen that the range can be widened for all strains. Those which originally grew at high salt concentrations (over 15 to 18 per cent) could be made to grow at as low as 12 per cent (below half saturation) without lowering their upper limit. The few on the other hand that originally did not grow above 21 or 24 per cent salt could be made to grow at saturation and at the same time their lower limit could be extended. None of the strains could be adapted to less than 6 per cent salt.

TABLE II. Salt tolerance of 25 red halophilic strains before and after adaptation to high and low salt concentrations.

% NaCl	27-21	27-18	27-15	27-12	27-9	27-6	24-12	21-15	Total
Original number	2*	5	9	4			2	3	25
Number after adaptation			1	17	2	5			25

*Although originally growing at 18.4% or a little higher, these at first showed practically no growth at 18%.

To summarize, of the original twenty-five strains, twenty grew at concentrations as high as 27 per cent (saturation), two had a maximum salt tolerance of 24 per cent, and three a maximum of 21 per cent. After adaptation all strains grew at 27 per cent. Only six of the original strains grew at concentrations down to 12 per cent, but after adaptation 24 strains grew at this concentration and five strains even in as low as 6 per cent.

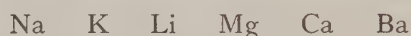
Growth, of course, is slower at the limits than in the middle of the range in salt concentration in all cases. The two *Sarcina* strains (isolations from seawater, Hess 1942a) showed an initially narrow range—15 to 21 per cent—but through

adaptation the range could be widened to 12 to 27 per cent salt. Lochhead (1934) found sarcina strains to have a lower optimum salt concentration, "20 to 24 per cent," than the *Serratia* strains, "28 to 32 per cent."

REPLACEMENT OF CATIONS

A considerable literature exists on the effects of various cations on the viability and metabolism of non-halophilic bacteria. Shaughnessy and Winslow (1927) and Winslow and Dolloff (1928) working with *Es. coli* reported that the effects upon viability are quantitative, rather than qualitative, i.e. the cations differed in degree rather than in kind. This applies to the stimulating action of the salts in low concentrations, as well as to their inhibiting action at high concentrations.

In general, it has been found that, based upon the effects of the salts, the cations can be arranged in a definite series of increasing inhibitory effect as follows:



Hotchkiss (1923) found concentrations of over 1.0 m. inhibitory for NaCl and KCl, over 0.5 m for LiCl and over 0.25 m for MgCl₂. Dumesh (1935) similarly found that isotonic solutions of different salts did not have the same effect on bacteria, but that their relative inhibitory effect was of the same order as mentioned above. Rubentschik (1926) obtained similar results with urea-splitting bacteria. It is noteworthy that this Hofmeister cation series is the same as that found by Gortner, Hoffman and Sinclair (1928) for the effect of cations upon the peptisation of proteins. Johnson and Harvey (1938) noticed a similar order in the effects of cations upon the viability, respiration and luminescence of marine bacteria.

In the case of halophilic bacteria the evidence in the literature is somewhat contradictory. Richter (1928) claims that sodium chloride in relation to growth of *Bacterium phosphoreum* acting as regulator of osmotic pressure can be replaced by any of a large series of salts. Baumgartner (1938) has shown this to be true for an anaerobic obligate halophilic organism (non-pigmented) isolated by him. He found on testing that several of such salts form a Hofmeister cation series in respect to concentration for maximum growth. Schoop (1935), on the other hand, showed that for obligate halophiles NaCl is essential, i.e. neither the Na ion nor the Cl ion alone, nor suitable osmotic pressure alone were sufficient to maintain growth of obligate halophiles, whereas facultative halophiles would grow under these circumstances.

In the present test on three strains of red halophiles and a strain of a facultative pink halophile (isolated from solar salt), skim-milk salt agar media were prepared containing 0.25 to 5.0 m NaCl, 0.25 to 4.0 m KCl, 0.25 to 3.0 m LiCl and 0.25 to 1.0 m MgCl₂, CaCl₂ and BaCl₂ respectively. All media were adjusted to pH 7.1 to 7.3.

The differences (table III) in the results with the two groups are striking and support Schoop's results, rather than Baumgartner's. Sodium chloride appears to be essential for the growth of these halophilic organisms. According to Golikova (1930) the bacterial cell of halophiles contains a large percentage of

TABLE III. Salt tolerance of halophilic bacteria.

Salt	Molar concentration					
	0.25	0.5	0.75	1.0	1.5	2.0
<i>Three red halophiles</i>						
NaCl.....	—	—	±	+	+	++
KCl.....	—	—	—	—	—	—
LiCl.....	—	—	—	—	—	—
MgCl ₂	—	—	—	—	—	—
CaCl ₂	—	—	—	—	—	—
BaCl ₂	—	—	—	—	—	—
T. Island.....		—		—	++	++++
<i>One pink facultative halophile</i>						
NaCl.....	+++	+++	+++	+++	+++	+++
KCl.....	+++	+++	+++	++	+	+
LiCl.....	++++	++++	++++	—	—	—
MgCl ₂	+++	++	±	—	—	—
CaCl ₂	+++	+	±	—	—	—
BaCl ₂	—	—	—	—	—	—
T. Island.....		+++		+++	+++	++++

Salt	Molar concentration					
	2.5	3.0	3.5	4.0	4.5	5.0
<i>Three red halophiles</i>						
NaCl.....	+++	+++	++++	++++	++++	++++
KCl.....	—	—	—	—	—	—
LiCl.....	—	—	—	—	—	—
T. Island.....	++++	++++		++++		++++
<i>One pink facultative halophile</i>						
NaCl.....	++	++	+	+	±	—
KCl.....	—	—	—	—	—	—
LiCl.....	—	—	—	—	—	—
T. Island.....	++++	++++		++		+

sodium chloride, to which he attributes their faculty to grow in a saturated salt medium. It may at the same time limit the salts usable in media to sodium chloride. On the other hand it has been shown by Gibbons (unpublished data) and by Hess (1942b) that traces of magnesium and calcium in presence of high sodium chloride concentrations as they are found in solar salts, increase the stimulating action and decrease the inhibitory action.

The effect of the various cations on the facultative halophilic strain shows that they can be arranged in a Hofmeister cation series in respect to their inhibitory effect.

SUMMARY

The temperature range, heat resistance, relative humidity range, effect of ultra-violet light and the salt tolerance of 25 strains of red halophilic bacteria have been studied.

Growth is stopped below 15° and above 55°C., indicating an unusually wide temperature range.

Optimum relative humidity and relative humidity range (70 to 85 per cent) are lower than for non-halophilic micro-organisms.

Heat resistance is low, less than 2 minutes' exposure to 98°C. when suspended in 24 per cent saline.

The organisms are exceptionally resistant to ultra-violet light (Westinghouse Sterilamp, 2537 Å units, at 10 cm. distance), as compared with non-halophilic micro-organisms, some surviving 120 minutes' exposure.

Through gradual adaptation all strains could be made to grow in skim milk-salt agar containing 27 per cent of NaCl (saturation). The lower salt limit could also be increased in all cases but none grew below 6 per cent NaCl.

Replacement of Na ion by K, Li, Mg, Ca and Ba inhibited growth of halophilic strains at all concentrations tested, whereas facultative halophilic bacteria would grow on all but the BaCl₂ media at the lower concentrations. For the latter organisms the cations could be arranged in a Hofmeister series in respect to their inhibitory effect.

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Studies on Salt Fish

X. Effect of Disinfectants and Preservatives on Red Halophilic Bacteria

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ABSTRACT

Fumigation with formaldehyde or sulphurous acid vapours was found effective against directly exposed red halophiles, using 10 oz. (283 g.) of the former or 5 lb. (2.268 kg.) sulphur per 1,000 cu. ft. (28.3 cu. m.) space. Formaldehyde solution 1:1,000 stopped growth in one minute, in absence of protein.

Curing salt mixtures containing 2 per cent sodium acid phosphate and 0.25 per cent sodium benzoate stopped growth of red halophiles as well as brown halophilic dun molds. Dips in preservative solutions are not effective.

It has been shown (Gibbons 1936) that red halophilic bacteria of the genera *Sarcina* and *Serratia* are responsible for the reddening of salt fish and that the common source of these organisms is solar salt. For the control of reddening, one must consider the measures that lead to the prevention of contamination of the fish with red halophiles, and the measures that cause an inhibition of the growth of red halophiles on contaminated fish. The former, when fully effective, will make the second unnecessary.

In order to prevent contamination, all potential sources must be eliminated. This involves use of salt free from red bacteria (uncontaminated mined or sterilized solar salts) and the sterilization of all fish-curing plants, boat holds, salt bins, and salt fish warehouses, which, once contaminated, act as secondary sources of contamination for all salt and salt fish passing through them. Such disinfectants have been studied (p. 18).

Owing to the great difficulty of gaining complete control in a short time over reddening by prevention of contamination, it is necessary to investigate possible means of inhibiting growth of the bacteria with which the salt fish have become contaminated. Some of the factors affecting the growth of these bacteria have been already dealt with (Hess 1942), namely temperature range, heat resistance, relative humidity range, ultraviolet light and salt tolerance. There remains the use of chemical preservatives (stabilizers), which has received attention from many workers. They provide the only means—apart from canning—of insuring permanent inhibition of bacteria in spite of any subsequent exposure to contamination. It is generally agreed (Hess 1939) that chemical preservatives should

be used only when other methods of preservation are not feasible, or inefficient. Their effectiveness for red halophiles has been studied (p. 20).

DISINFECTANTS

Among the disinfectants that have been advocated for the sterilization of plants and boats are lye, chlorine solutions, formaldehyde, sulphurous acid and lime water.

LYE (COMMERCIAL SODIUM HYDROXIDE)

One ml. portions of heavily contaminated brine were added to 10 ml. each of different lye solutions, and samples withdrawn at one-minute intervals and inoculated into skim-milk salt agar plates (Hess 1942). Survival was observed after incubation at 37°C. for 30 days. Killing times were as follows:

Lye (%).....	2	1.3	1	0.67	0.5
Time (min.).....	2	4	6	7	14

McCulloch (1933) also found a 2 per cent solution of lye effective, even in the presence of organic matter.

CHLORINE

As with the lye solutions, 10 ml. each of chlorine solutions of different strengths were inoculated with red organisms and samples withdrawn at half-minute intervals. Killing times were as follows: 0.5% available chlorine, 1 minute; 0.2% available chlorine, 1½ minutes.

Inoculated skim-milk salt agar plates were washed with chlorine solutions for one minute, rinsed with sterile brine and incubated at 37°C. Solutions containing 0.2% available chlorine were ineffective, but 0.5% chlorine solutions stopped growth on the plates.

FORMALDEHYDE

Formaldehyde is used extensively in the fresh fish industry, in the treatment of boat holds in particular, since it was advocated by Bedford (1935) to eliminate the yellowish bacterial discoloration of halibut.

To test the formaldehyde tolerance of red halophilic bacteria, formaldehyde was added directly to skim-milk salt agar plates before the agar was poured into them. The plates were inoculated with four strains of red halophiles each, and incubated at 37°C.

Formaldehyde (%).....	0.1	0.02	0.01	0.005	0.003	0 (control)
Growth.....	—	—	±	+	+	+

In another test skim-milk salt agar plates were inoculated over their whole surface with emulsions of red halophiles. After allowing the surface to dry, one drop of a formaldehyde solution was placed in the centre of each plate, and the latter incubated at 37°C. Within three weeks no growth had appeared on the plates to which one drop of a 4% solution had been added, while the surface of the plates receiving one drop of 0.4 or 0.04% solution showed growth, except in

the centre of the plates where circular areas of 30 mm. and 12 mm. diameter, respectively, remained free of growth.

Finally, emulsions of four strains each were spread on sterile cover glasses and allowed to dry, following the technique of Jensen and Jensen (1933). The cover glasses were then dropped into formaldehyde solutions of different strengths, removed at regular intervals, and allowed to drain. An impress was made on skim-milk salt agar plates, and the latter incubated at 37°C., with the following results:

Formaldehyde (%).....	0.4	0.1	0.04	0.01	0.004	0.001
Exposure (sec.) . growth...	30	30	60	120	120	360
. no growth	45	60	120	180	240	480

These results, when compared with those reported in the first paragraph, confirm the generally known quenching effect of protein on the formaldehyde action, e.g., whereas 0.005% formaldehyde in the presence of skim milk did not prevent growth in the first experiment, in this test, in the absence of protein, 0.004% formaldehyde stopped growth after exposure of less than four minutes.

Exposure of inoculated plates to direct sprays of formaldehyde solutions showed great variations in the resistance of different strains; while the growth of most strains was stopped after five seconds of spraying with a 1% formaldehyde solution, some resistant strains survived ten seconds' spraying with a 5% solution.

In experimental fumigation tests an air-tight room of 360 cu. ft. (10.2 cu. m.) was used, and formalin was mixed with potassium permanganate in the proportion of 10 oz. (283 g.) of the former to 5 oz. (142 g.) of the latter per 1,000 cu. ft. (28.3 cu. m.) immediately before closing the door of the room. Inoculated skim-milk salt agar slants in plugged test tubes and uncovered plates were exposed to the developing formaldehyde vapours in various parts of the room. After 20 to 24 hours the room was opened, the tubes or plates removed and incubated at 37°C. On all exposed uncovered plates growth was stopped, while in some of the cotton-plugged test tubes growth occurred at the base of the slants. Plates which had been completely covered with pieces of wood $\frac{1}{2}$ inch, $\frac{3}{4}$ inch and 1 inch (2.5 cm.) thick during the exposure,—the edges of the plates being set in vaseline—showed growth of the test organisms in all cases, thus indicating that formaldehyde vapours have no great powers of penetrating through dry wood, or cotton batting.

SULPHUROUS ACID

Sublimed sulphur was burned in the same room described above, in the proportion of 5 lb. (2.268 kg.) of sulphur per 1,000 cu. ft. (28.3 cu. m.) of space. The room was opened after 20 to 22 hours and the exposed, previously inoculated slants and plates removed and incubated at 37°C. As with the formaldehyde vapours, growth in all uncovered plates was stopped, while it occurred at the base but not at the top of the slants. Penetration of the sulphur vapours was better than that of formaldehyde in the case of wood, as in some cases plates covered with $\frac{1}{2}$ inch to 1 inch thick wood showed no growth, while in other cases only the $\frac{1}{2}$ inch wood allowed penetration, stopping growth, but $\frac{3}{4}$ and 1 inch thick wood did not.

PRESERVATIVES

As pointed out in the introduction, chemical preservatives may be indicated, as a last resort, to inhibit the growth of red halophilic bacteria on material already contaminated or to prevent growth after subsequent contamination. The choice of preservatives to be used depends to a large extent upon the material to be preserved. For non-edible products, such as salted hides and intestines, a wider choice is possible than for foods, where the choice is limited by considerations of the effects of the preservatives not only on the red halophiles but also on the flavour and appearance of the food and on the health of the consumer.

The effects of a large number of preservatives upon red halophiles have been studied in a number of countries. The effective use of boric acid, potassium nitrate, sodium carbonate, sodium hyposulphite, sodium chlorobenzoate, sodium chlorocinnamate, sodium sulphobenzoate and sodium benzoate has been reported by LeDantec (1891), Bitting (1911), Boury (1934), Fillon (1924), Hanzawa and Takeda (1931) and Cloake (1933). The majority of these substances, while at one time permissible as preservatives, are today excluded as such by our Pure Food Laws.

Preservatives may act in different ways, as buffers, through osmotic effects, or through specific chemical interactions with the bacterial protoplasm.

INORGANIC BUFFERS

It was shown by Harrison and Kennedy (1922) that growth of red halophiles is inhibited between pH 5.6 and 5.2 and between pH 8.6 and 9.0. Gibbons (1936) also found pH 5.5 and 9.0 to be the lower and upper limits of hydrogen-ion concentration for a number of red halophilic *Sarcina* and *Serratia* strains.

The brines formed in the curing of codfish with various solar salts were found to have a pH range of 6.4 to 6.6, which is approximately the optimum hydrogen-ion concentration for most red halophiles. The use of acid phosphate buffers was tested by mixing sodium dihydrogen phosphate and potassium dihydrogen phosphate with sodium chloride each in the proportion 1:19 and using the mixtures to cure contaminated fish. The resulting brines reached a pH of 5.1 for sodium dihydrogen phosphate and 5.5 for potassium dihydrogen phosphate. No growth of red halophiles occurred during incubation at 37°C. for three months.

The buffering of fish muscle by means of dipping in a saturated salt brine which had been buffered to approximately pH 3.2 with sodium dihydrogen phosphate required three hours' immersion to increase the hydrogen-ion concentration at the fish surface to pH 5.2.

Using a mixture of 5 per cent sodium carbonate in solar salt to cure fish a buffering effect at approximately pH 10 was obtained and growth of red halophiles on contaminated fish was prevented.

ORGANIC BUFFERS

Nadeau (1940) has shown that bacterial growth and trimethylamine production in fresh cod press juice at 0°C. could be practically inhibited by buffering the juice with organic acids (citric, lactic, tartaric) at pH 5.0 and lower. A con-

centration of approximately 0.3 per cent acid was required to obtain a pH of 5.0 in the juice.

Nunheimer and Fabian (1940) observed that organic acids exerted a germicidal and antiseptic effect disproportionate to the hydrogen-ion concentration produced, and concluded that the effects are due to other factors besides the H-ion, such as the un-ionized molecule or the anion, or both. Erickson and Fabian (1942) also found that based upon the H-ion concentration the germicidal effect of such acids was of the order: acetic acid > citric > lactic, but based upon their concentration by weight the order was: lactic > acetic > citric, although their evidence seems rather too weak for drawing such general conclusions.

The effects of several such organic acids upon red halophiles were tested by adding them to laboratory media, salt fish broth or skim-milk salt agar, in different concentrations. The sterilized media were inoculated with a number of strains, and incubated at 37°C. to determine whether there would or would not be growth.

TABLE I. Effect of organic acids on growth of red halophilic bacteria.

Acid	Dissociation constant	Concentration of acid in salted (22.5%) skim-milk agar				
		0.01%	0.02%	0.04%	0.1%	0.2%
Propionic.....	1.4×10^{-5}	+	+	—	—	—
Acetic.....	1.86×10^{-5}	+	+	+	—	—
Benzoic.....	6.6×10^{-5}	—	—	—	—	—
Lactic.....	1.38×10^{-4}	—	—	—	—	—
Citric.....	8×10^{-4}	—	—	—	—	—
Tartaric.....	1.1×10^{-3}	—	—	—	—	—

The results (table I) show citric and lactic acids to be more effective in preventing growth than is acetic acid, as was found by Erickson and Fabian (1942) for a number of bacterial species.

It is apparent that salted skim-milk agar is in itself not as good a buffer as codfish press juice, as prepared by Nadeau, since relatively small acid concentrations produced low enough pH values to inhibit growth. This fits in well with the findings of Gorbunov (1937), who observed that the additions of low concentrations of acetic acid during salting did not prevent the growth of red halophiles, and attributed this to the high buffer action of fish proteins.

Experiments using mixtures of contaminated solar salt and acetic acid for curing fish confirmed this. At least three parts by weight of acetic acid per 97 parts of solar salt were required to prevent growth of red halophiles; this caused yellow discoloration of the fish muscle.

Of the acids tested, propionic was found the most suitable, affecting odour and appearance of the treated media least of all. Propionic acid has also been found to inhibit growth and sporulation of halophilic brown molds of the genus *Sporendonema* in concentrations of 0.1 to 0.2 per cent at pH 4.0 to 4.5 (Frank and Hess 1941).

Dipping contaminated fish for five minutes in approximately half-saturated brine containing propionic acid in at least 0.5 molar concentration was found necessary to prevent growth of halophiles, according to Frank (unpublished data); with shorter dips (30 seconds) a minimal molar concentration of 0.8 was necessary. To what extent the effect of such treatment is a buffering action or a specific chemical reaction was not determined. Frank and Hess (1941) pointed out, however, the effect of hydrogen-ion concentration upon the specific effects of preservatives such as propionic and butyric acid and their calcium and sodium salts.

SODIUM BENZOATE

The effective use of sodium benzoate in controlling the growth of red halophiles has been reported by Bitting (1911), Boury (1934) and Hanzawa and Takeda (1931).

Experiments using the preservative mixed with contaminated solar salt in the curing of fish showed that at least 0.5 per cent by weight was needed to inhibit growth of red halophiles at room temperature, but 2 per cent to prevent growth at 37°C., the resulting pickle reaching a reaction of approximately pH 6.5.

The effect of sodium benzoate could be increased by increasing the hydrogen-ion concentration as shown by Frank and Hess (1941) and Cruess and Richert (1929). Since, as mentioned above, either a salt mixture buffered with 5 per cent sodium dihydrogen phosphate or a salt mixture containing 2 per cent sodium benzoate prevents the growth of red halophiles, the combination of the two, i.e. the buffering of a much weaker sodium benzoate-salt mixture, would be as effective in inhibiting bacterial growth. In fact, buffering with only 3 per cent sodium dihydrogen phosphate enabled a reduction of the sodium benzoate content of the mixture to 0.25 per cent. Similarly a mixture of sodium benzoate, sodium dihydrogen phosphate and solar salt in the proportions of 3:20:20, sprinkled over contaminated fish and resulting in less than 0.1 per cent sodium benzoate in the total weight of the fish, inhibited growth of red halophiles.

SODIUM NITRITE

Tarr and Sunderland (1940a) found that ice containing 0.1 per cent sodium nitrite is much more effective in inhibiting growth of bacteria on dressed fish than is ice containing an identical proportion of benzoic acid, and that sodium nitrite and potassium nitrite in 0.01 per cent concentration causes a much greater inhibition in bacterial spoilage of fresh fillets than does sodium benzoate or benzoic acid (1940b). Later Tarr (1941) noticed that the effectiveness of 0.02 per cent concentrations of sodium nitrite depends to a large extent on the reaction of the media in which a series of test organisms were studied. At pH 7 or above growth was not inhibited significantly, while between pH 5.7 and 6.5 growth of most cultures was largely inhibited or completely stopped.

Ten strains of red halophiles were tested on salted skim-milk agar media containing 0.005, 0.01, 0.02, 0.05, 0.1 and 0.2 per cent NaNO_2 respectively at a pH of approximately 5.5; 0.1 per cent of the preservative was the lowest concen-

tration found to inhibit growth, the media becoming discoloured (yellowish brown). This concentration is much higher than that permitted by our Pure Food Laws.

SUMMARY

The action of commercial disinfectants against red halophilic bacteria, for use in the sterilization of salt fish plants and boats, has been studied.

A 2 per cent solution of lye was effective in two minutes and a chlorine solution containing 5,000 ppm of available chlorine in one minute.

A formaldehyde solution 1: 1,000, in absence of protein, stopped growth in one minute. Fumigation (10 ounces formalin per 1,000 cu. ft. of space) killed all red halophiles directly exposed but did not affect those protected by cotton batting or wooden boards $\frac{1}{2}$ to 1 inch thick.

Fumigation with sulphurous acid (5 pounds of sublimed sulphur burned per 1,000 cu. ft.) had similar results with slightly higher penetrating power through wood.

The use of preservatives mixed with the curing salts to inhibit growth of red halophiles on contaminated fish has been investigated. Of the mixtures tested, one, containing 3 per cent sodium acid phosphate and 0.25 per cent sodium benzoate, was found most effective in inhibiting both red halophilic bacteria and brown halophilic "dun" molds.

Dips of contaminated salt fish in preservative solutions were not very successful.

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Life History of *Lepeophtheirus salmonis*

BY H. C. WHITE

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ABSTRACT

Heavy infestations of this copepod in various developmental stages occur on *Salmo salar* and *Salvelinus fontinalis* at Moser River, N.S. Males and females occur in about equal numbers. Metanauplius and chalimus stages were present on the fins of the hosts. The male and four of the developmental stages are described and figures given. The parasite feeds on the skin of its host.

This parasitic copepod is widely distributed, occurring on several species of Salmonidae on the north Atlantic coasts of Europe and America and also in the North Pacific ocean. We have found individuals in small numbers on salmon in the bay of Fundy, somewhat more numerous in the gulf of St. Lawrence, and very abundant on salmon and sea-running brook trout returning to the Moser river on the southeast coast of Nova Scotia. They are, however, unable to complete their life cycle on the trout of that region as the trout remain in salt water for but two months and the parasites die when the fish return to fresh water (White 1941). In 1939 the infestation of the salmon at Moser River was sufficiently severe to cause the death of some of the salmon (White 1940). Since the returning fish carried large numbers of the parasite in various stages of development, material was collected for a study of its life history.

OCCURRENCE ON HOST

Wilson (1905) states that "The males are very scarce". Scott (1913) says, "Males appear to be comparatively scarce". In our collections from Moser river in 1940 there were 392 of the parasites in the adult form, of which 175 or 45% were males. One lot scraped from a salmon grilse contained 23 females and 23 males. Other lots taken from "trout" contained 14 females and 21 males, and 11 females and 20 males respectively. It would appear that the males and females occur in about equal numbers. Since the males are much more motile than the adult females and are not so confined to particular areas of the fish, it is probable that they have been largely overlooked by collectors.

Our samples were collected mostly by scraping random samples from the body of the living fish. One sample from the head of a grilse yielded five indi-

viduals, all males. Females were dominant in samples taken from the backs of the fishes. Wilson's single male was reported to have come from the gills of a salmon. We have examined many heavily infested fish but have not found this parasite in any stage within the gill cavity. Young, of both sexes, in the adult form range over much of the body of their host (White 1940, fig. 2), but the metanauplius and chalimus stages are largely confined to the fins.

THE FEMALE

Large mature females with their long egg strings are generally found on the salmon just behind the dorsal fin or along its base, or on the ventral side, just posterior to the anal fin. Wilson (1905) gives the length of the mature female as 18.2 mm. with egg strings 53 mm. The largest in our collection from Moser

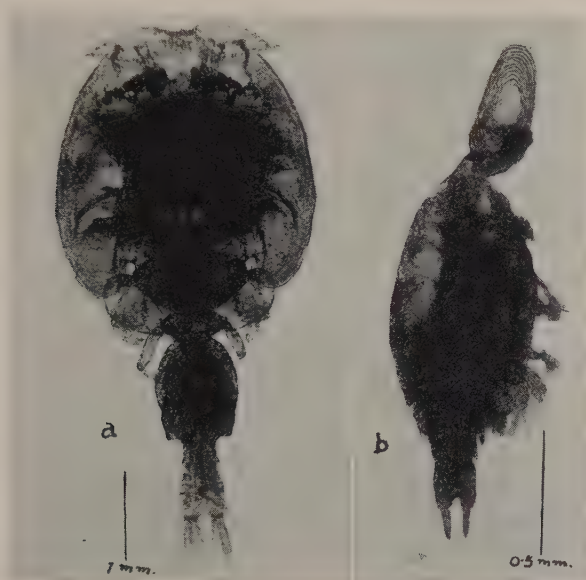


FIGURE 1. *a*,—Adult male; *b*,—second chalimus stage attached by frontal filament to scale of trout (*Salvelinus fontinalis*).

river are 16 mm. long with egg strings 38 mm. These were among mature females on salmon taken in a commercial net on June 7, 1940. The eggs at this time were dark brown, indicating that they were near the hatching stage. Since these were the largest adults in our collection and no immature individuals were found on the salmon taken at that time, it is evident that they pass the winter in the adult stage and do not reproduce during the period of cold water.

MALE

The male has been described by several authorities and has been figured by Wilson (1905, pl. XXIV), who gives the size of his specimen as 6 mm. in length. Sexually mature males from our collections varied in size according to the time

taken. Nineteen sexually mature males taken July 31 averaged 5.35 mm. Twenty-one taken August 20 averaged 5.46, and three males collected June 7 averaged 6.7 mm. Although all were sexually mature, only those taken in early June which had lived over the winter had attained their full growth.

The males of our collection do not entirely conform to the descriptions given either by Wilson (1905) or Scott (1913) and differ markedly from the figure given by Wilson.

A sexually mature male (fig. 1a) had the following dimensions in mm.: 5.6 in length to the end of the anal laminae; carapace 3.3 long; 2.9 wide; free segment 0.4 long and 0.7 wide; carapace overlapping free segment 0.1; genital segment 1.2 long and 0.9 wide; abdomen 0.6 long; anal laminae 0.2 long.

The frontal plates are narrow. The carapace is elliptical with the greatest width at one-third its length from its posterior edge. The posterior sinuses are narrow and converge anteriorly. The free segment is wider than long and the fourth pair of swimming legs, which are slender, is attached at the middle of the segment. The genital segment is ovoid and is widest at about one-third its length from its anterior end. It narrows abruptly at its posterior end where, at a slight constriction, it joins the abdomen. Just above the posterior end of the genital segment there is a pair of obtuse vestigial legs each bearing two minute spines. A second minute leg with a terminal joint is situated at the anterior ventral edge of a depression above the base of the larger vestigial legs. The kidney-shaped spermatophore receptacles are readily recognized in the mature living males and are shown as dark a patch in the figure.

The abdomen has, anteriorly, a short narrow imperfectly formed segment followed by a wider segment which expands slightly posteriorly. The anal laminae are large and are but slightly longer than their greatest width and bear four plumose setae. The appendages, excepting the second antennae, are similar to those of the female. The second antennae are much stouter than those of the female (fig. 2g) and bear a large chitinous pad (fig. 2f).

EGGS

The eggs are relatively small and are extremely flattened in the egg-strings. Eggs nearing the hatching period were found early in June and also late in August, but it is probable that eggs hatch throughout the summer and fall.

NAUPLIUS

No nauplii were obtained. They probably resemble those of closely allied species such as *L. edwardsii* (Wilson 1905, p. 540, fig. 39).

METANAUPLIUS

Metanauplii in their final moult were found attached by their strongly-hooked second antennae to the fins, especially the dorsal fin of "trout" and salmon (fig. 2a, unrelaxed specimen preserved in formalin solution).

The carapace is three-fifths of the total length and is two-thirds as broad as long. The rest of the body is composed of three definite free thoracic segments and the abdomen.

The appendages shown in the figure consist of large first antennae with two joints of about equal size, the terminal joint having six long plumose setae and several smaller ones. The second antennae are three-jointed and terminated by strongly hooked claws. The second maxillipeds are large and project laterally beyond the carapace. The first thoracic segment is covered by the carapace.

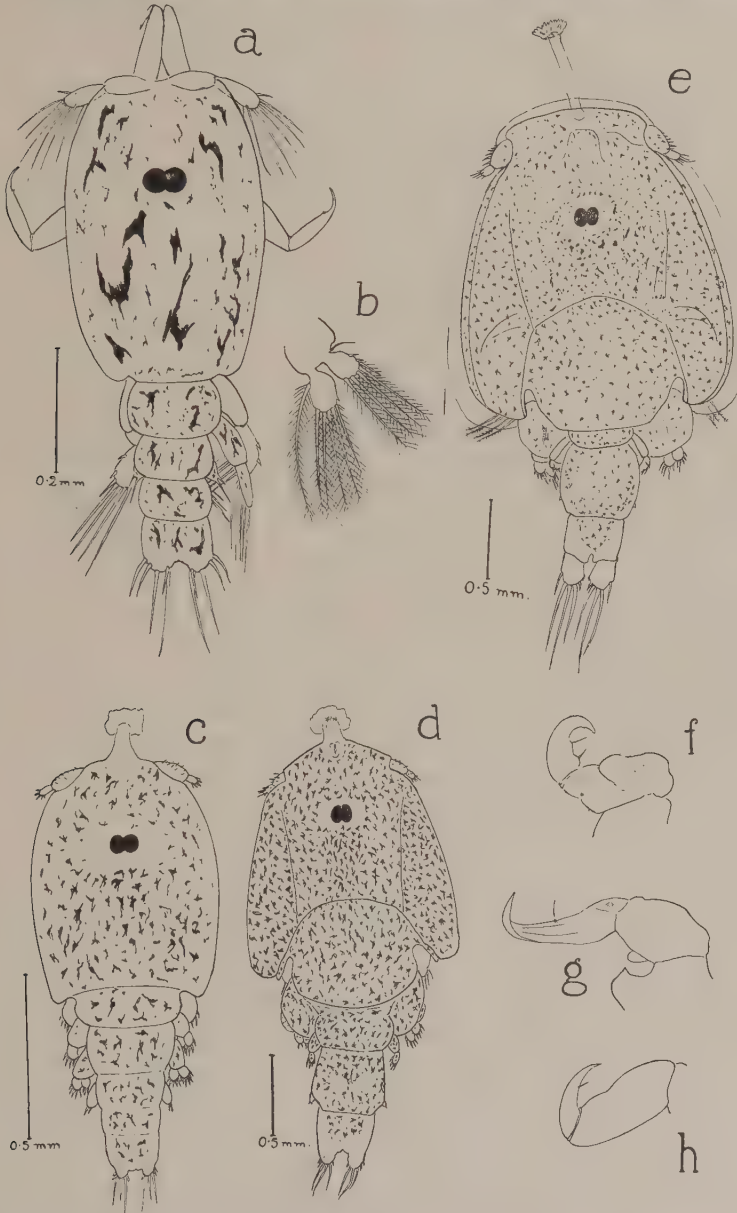


FIGURE 2. *a*,—*Metanauplius* 0.8 mm. long; *b*,—first swimming leg of *metanauplius*; *c*,—first chalimus stage 1.25 mm. long; *d*,—second chalimus stage 2.25 mm. long; *e*,—final chalimus stage 3 mm. long; *f*,—second antenna of male; *g*,—second antenna of female; *h*,—second maxilliped of male.

The first and second swimming legs are biramose and have flattened endopods and exopods bearing long plumose setae (fig. 2b). The third swimming leg is represented by a small protuberance bearing two setae. No trace of the fourth swimming leg could be found. The abdomen is short. The anal laminae are short and broad and bear two long and three short plumose setae. The eyes are well developed. The pigment, particularly of the carapace, tends to be in large dense patches.

THE CHALIMUS STAGES

Salmon and "trout" returning from the sea at Moser river carried large numbers of the parasites in the chalimus stages (fig. 1b). These were present to some extent on all the fins (especially on the dorsal fin) and extended down on the skin along the base of the fin.

The earliest chalimus stage found (fig. 2c) is 1.25 mm. long. The first antennae have attained much the form found in the adult. The first joint (according to Wilson 1905) has become one of the frontal plates, but has added a small terminal joint bearing a number of short setae. The second antennae and the second maxillipeds are now relatively short thick prehensile organs. There are four pairs of thoracic legs, although the fourth pair is represented by only a bud-like joint bearing two short setae. The anal laminae are short and bear six setae, three of which are very short. The tergum of the second thoracic segment is now definitely fused with the carapace and forms the median lobe. The third and fourth segments are free. The rest of the body consists of two indistinctly separated segments, being the genital segment and the abdomen. The pigment is arranged in smaller patches than in the metanauplius.

The second chalimus stage (fig. 2d) measured 2.25 mm. The appendages, with the exception of the fourth thoracic legs, are much like those of the adult form. The anal laminae are longer than in the previous stage. The tergum of the second thoracic segment has become the thoracic plate, which covers three of the fused thoracic segments but not the fourth. The genital segment is much enlarged and is definitely separated from the abdomen and has a pair of small vestigial legs.

There are several other chalimus moults which show slight changes. The final chalimus stage (fig. 2e) is 3 mm. in length. The anal laminae have become longer and have but five setae, three long and two short. The carapace is wider and more like that of the adult, covering the greater part of the body. The fourth or free segment is relatively much smaller and is partly covered by the median lobe. The genital segment is much enlarged and has a pair of vestigial legs. In this stage the frontal filament is relatively longer than in the previous chalimus stages.

After the chalimus stages there are numerous moults before the parasites attain their maximum size.

FOOD

There has been considerable controversy among those studying the Caligidae concerning the nature of their food. Some authorities maintain that they feed only on mucus. Wilson (1905) maintains that they feed upon blood.

Whatever may be the case with other Caligidae, *Lepeophtheirus salmonis* certainly feeds upon the skin tissues and even upon the subcutaneous tissues. It may, however, and we believe that it does, feed also upon mucus and blood or lymph. We have pointed out in a previous paper (White 1940) to what extent it erodes the skin of its hosts. Since then we have been able to find bits of fish skin with melanophores in their digestive tracts and also we have observed them taking into their mouths minute pieces of chopped skin which we have fed them.

During the chalimus stages, when attached to the fin of the fish the young parasites swing about on their frontal filaments when not feeding, but when feeding attach themselves by their prehensile appendages and eat the skin in a small arc around the point of attachment of the frontal filament. On heavily infested fins much of the fin surface may be eaten away.

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Peculiar Variants of the Agonid Fish *Odontopyxis trispinosus*

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ABSTRACT

Two agonid fish from British Columbia are regarded as aberrant, perhaps mutant examples of *Odontopyxis trispinosus*. Their chief distinctive feature is the fusion, at some point before the dorsal fin, of the dorsal shields and keels, which are normally paired in this region. In this respect only, they approach *Bothragonus swanii*. The variants show a tendency toward aberrancy in some other characters, but in most respects are typical of *Odontopyxis trispinosus*.

Almost ten years ago Mr. G. V. Wilby, of the Pacific Biological Station, at Nanaimo, British Columbia, sent me for study an agonid fish (fig. 1) which seemed to represent a new genus and species. It shared many characters with *Odontopyxis trispinosus* Lockington, but was very distinctive in other respects. A second aberrant example (fig. 2B) of the *Odontopyxis* type, collected by Mr. Wilby in 1934, agreed with the first specimen in some characters but was peculiar in other features. It was hoped that other fish like either of these two might be discovered, but they remain unique.

On renewed, detailed comparison, the two aberrant specimens are now identified as *Odontopyxis trispinosus*. In most characters the agreement is satisfactory, and in the variant features the two examples in question are not as consistent as would be expected, were they members of a new species. The chance remains that they represent two new species of a new genus, but this possibility now seems remote.

I am indebted to Dr. W. A. Clemens and Mr. G. V. Wilby for the privilege of studying these interesting variants, to Grace Eager for the beautiful drawing reproduced as figure 1, and to Clarence Flaten for the photographs on which figure 2 is based.

ANTERIOR FUSION OF THE DORSAL PLATES AND KEELS

The outstanding feature common to the two variants is the fusion before the dorsal fin of the dorsal ridges and rows of bony shields. At the point of fusion the normally concave anterior back (fig. 2A) becomes convex, just as the back behind the second dorsal fin in normal specimens becomes elevated, where the dorsolateral ridges of the anterior part of the body fuse to form a mid-dorsal keel.

In details, however, the anterior fusion of the dorsal keels is quite different. In the first aberrant specimen, hereinafter referred to as Variant 1 (dredged by J. L. Hart in Nanoose bay, British Columbia, on June 5, 1931), the fusion takes place at the fifth of the six pairs of predorsal bony plates (fig. 1). The modification is almost perfectly symmetrical, and the ridges are evenly curved toward the point of union. The fusion of the ridges is not quite complete, for the spines remain distinct, though closely approximated. The fifth (median) plate is not fused with the adjoining plates, but is slightly movable. Its posterior edge at the midline is extended into a concavity between the front edges of the paired (sixth) plates just in front of the dorsal. The body thus appears to be hinged on this ball-and-socket joint, which lies approximately over the anus. As a result the trunk and tail regions are independently movable to a small degree, instead of being firmly bound in a common casque. A complicated and apparently normal structure seems to be involved.

In Variant 2, collected by G. V. Wilby in Burrard inlet, at Second Narrows bridge, in 10 to 20 fathoms (18 to 36 m.) on February 1, 1934, the dorsal ridges are fused not far behind their origins (fig. 2, B). The ridges are well separated on the first pair of plates, and converge to a high point on the posterior edge of the second plate (which is median as a result of fusion). The third plate is also median, and bears a rounded keel which is not quite median, for it is nearly continuous behind with the dorsolateral keel of the right side. The sutures separating the three pairs of predorsal plates that follow run obliquely forward and to the right. The back is concave inside the V formed by the ridges in front of their fusion; it is also concave just behind the fused portion of the keel, but becomes almost flat at the origin of the dorsal fin. In this specimen the body is not hinged at the point of fusion of the keels; the only considerable motion takes place between the first pair of plates and the processes of the skull on either side of the occipital depression.

One of the most interesting features of the anterior fusion of the dorsal keels in the two variants is that such a fusion is diagnostic of the supposedly related but highly bizarre *Bothragonus swanii* (Steindachner) (fig. 2, C). This fact lends credence to the idea that such a systematic change may have been created in a single mutation.

The conclusion that the anterior fusion of the dorsal shields in the British Columbia specimens represents an anomaly is confirmed by the finding of a similar abnormality in one of the Japanese agonids.

EXSERTED PECTORAL RAYS

Another character in which the two variants agree is a marked exsertion of the lower pectoral rays. In this character, too, Variants 1 and 2 differ in detail; so do other specimens of *Odontopyxis trispinosus*, according to the following statement by Jordan and Evermann (1898, p. 2086): ". . . . pectorals with 14 rays, of which the lower 5 to 7 are exserted, the depth of the notches in the membranes variable, the 2 or 3 uppermost of these exserted rays nearly as long (sometimes as long) as the longest upper rays."

In Variant 1 the lower rays are rather strongly exserted, by reason of the

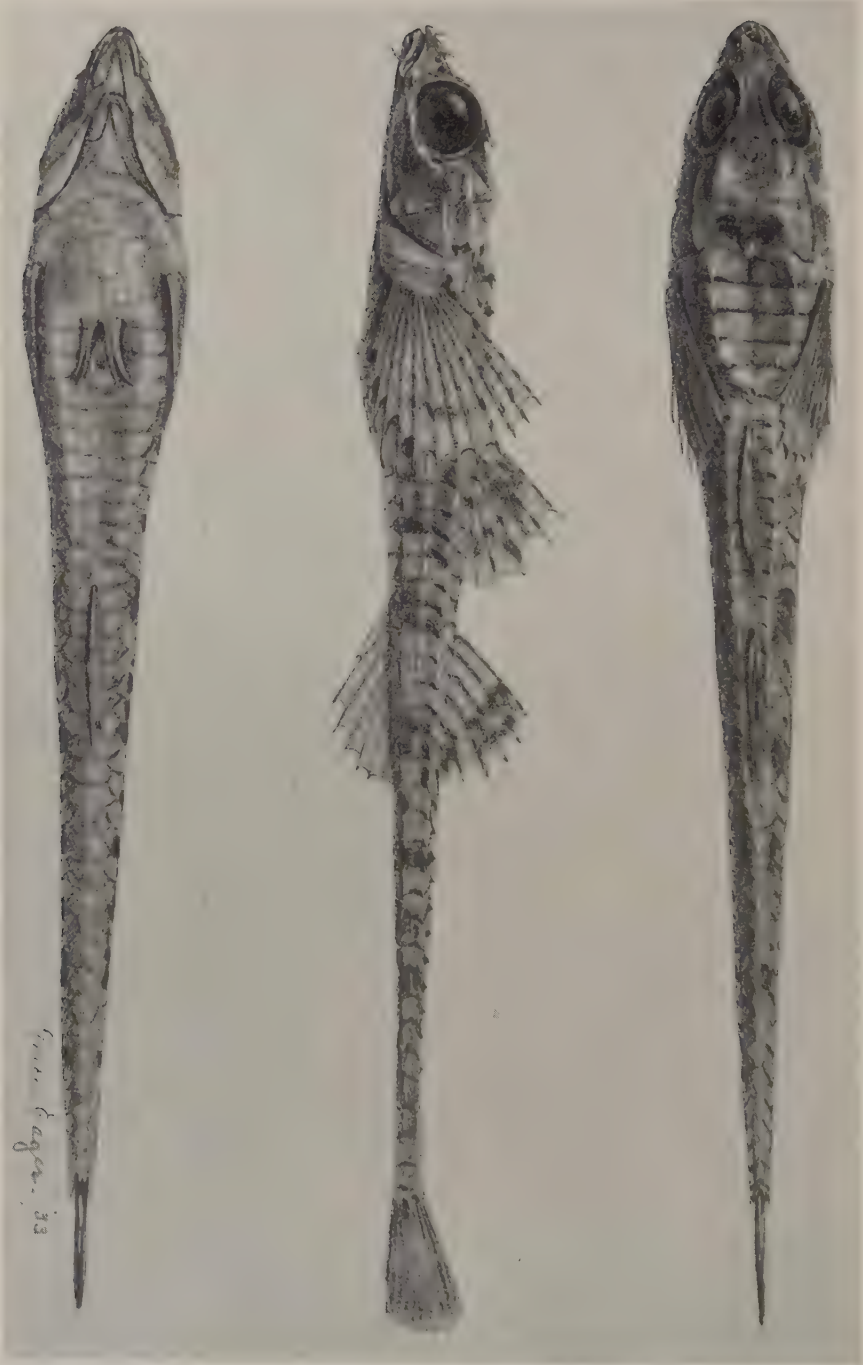


FIGURE 1. Dorsal, lateral, and ventral views of Variant 1 of *Odontopyxis trispinosus*. (Drawn by Grace Eager, staff artist of the University of Michigan Museum of Zoology, from a specimen 63 mm. in standard length, dredged by J. L. Hart in Nanoose bay, British Columbia, June 5, 1931)

deeply incised membranes. On the left side the fourth and fifth rays from below are subequal and extend about one-sixth of the orbital length beyond the next ray above (fig. 1). In the right pectoral the fourth ray from below is about as long as the longer upper rays, but the fifth ray extends fully one-third of the orbital length beyond the sixth ray. In none of the seven other specimens of *Odontopyxis trispinosus* at hand are the lower pectoral rays so notably exserted.

In Variant 2 the exsertion of the lower pectoral rays is next best developed. On the left side the sixth ray from below extends slightly beyond the fifth and seventh. On the right side the fifth ray passes beyond the fourth and sixth a little more than one-fifth the length of the orbit.

In another British Columbia specimen, normal as regards the dorsal keels (fig. 2, A), the lower pectoral membranes are almost as deeply incised as in the variants. In this fish the sixth and seventh rays from below are subequal, and extend a shade farther than the next rays above. In five specimens at hand from southern and central California the lower pectoral rays are less exserted than in any of the three from British Columbia. Possibly there is some geographical as well as individual variation in this character.

OTHER PECULIARITIES IN THE OSSEOUS PLATES

Except for the fusion of the plates of either side to form 1 or 2 median scutes before the dorsal fin, the number and arrangement of the dorsal plates in Variants 1 and 2 are not distinctive. Variant 1 has 12 pairs of plates in the ventral series from the pelvic fin to the front of the anal, whereas all other specimens at hand have 13; it has 6 pairs along the anal fin, as in only one of the seven other specimens (the others have $4\frac{1}{2}$ to $5\frac{1}{2}$ pairs). Variant 2 has 13 pairs of ventral plates before the anal fin and $5\frac{1}{2}$ pairs along the base of that fin, as in several of the other specimens.

In Variant 1 (fig. 1), but not in Variant 2, the scutes on the lower side of the head and on the breast exhibit some reduction in number, due to loss and to fusion. The lower part of the cheek on one side has only 1 plate, instead of the usual 2 (two of the seven other specimens, however, show the same variation). The Y-shaped gular patch has only 3 scales, 1 median and 1 in each arm; the seven other examples have 6 or 7 gulars. The triangular patch between the branchiostegals is armed with only 2 scutes (3 to 5, usually 5, in the others). The total number of plates on the lower side of the head is only 12—4 to 8 fewer than in any of the other seven fish. On one side of Variant 1 the 2 outer scutes of the third row are lacking (apparently through fusion with the corresponding scutes of the second row); none of the other specimens has these plates fused.

Considerable variation is evident in the number and arrangement of the bony shields in the several cross series on the breast, as detailed in table I. The first series is the only one showing no variations in number of elements; it is composed of 1 median and 2 lateral scutes. This set of 3 osseous plates is separated by a groove from the second series, as also from the branchiostegal patch, so that it is independently movable. The second series, which follows, and which extends laterally and upward along the edge of the shoulder girdle, is interrupted



FIGURE 2. Anterior dorsal views of *Odontopyxis* and *Bothragonus*. (Photographed by Clarence Flaten): A.—A British Columbia specimen of *Odontopyxis trispinosus*, 63 mm. in standard length, showing the normal arrangement of the bony plates and keels on the anterior back. B.—Variant 2 of *Odontopyxis trispinosus*; 67 mm. in standard length, collected by G. V. Wilby in Burrard inlet, British Columbia, on February 1, 1934. C.—*Bothragonus swanii*; 53 mm. in standard length, poisoned in a tidal reef pool near La Push, Washington, on June 25, 1926.

TABLE I. Number of osseous plates and of fin rays in *Odontopyxis trispinosus*.

	British Columbia			California				
	Variant 1	Variant 2	3	4	5	6	7	8
Bony plates								
<i>Dorsal row</i>								
Pair of plates								
Before 1st dorsal ..	4+1	4	6½	6	6	6	6	6
Along and between dorsals.....	14	14½	13½	14	14	13½	14½	14
Just behind 2nd dorsal.....	1	½	½	0	1	1½	½	1
At caudal base....	1	1	0	1	1	0	1	1
Total pairs.....	21	20	21	21	22	20	22	22
Median plates								
Before 1st dorsal...	1	2	0	0	0	0	0	0
Behind 2nd dorsal	15	14	15	15	15	15	15	15
Total median.....	16	16	15	15	15	15	15	15
Total dorsals.....	37	36	36	36	37	35	37	37
<i>Ventral row</i>								
Pairs of plates								
Before anal.....	12	13	13	13	13	13	13	13
Along anal.....	6	5½	5½	5	5½	4½	5½	6
Behind anal.....	1	½	½	0	½	1½	½	0
At caudal base....	1	1	0	1	1	1	0	1
Total pairs.....	20	20	19	19	20	20	19	20
Median plates								
Behind anal.....	16	15	16	16	16	16	16	15
Total ventrals.....	36	35	35	35	36	36	35	35
<i>Lower side of head</i>								
Lower cheek.....	2-1	2-2	2-1	2-2	2-2	2-2	2-2	2-1
Mandible.....	2-2	2-2	2-2	2-2	2-2	2-2	2-2	2-2
Gular patch.....	3	7	6	6	7	7	6	6
Branchiostegal patch.	2	5	3	4	5	5	5	5
Total (both sides) ...	12	20	16	18	20	20	19	18
<i>Breast (left-median- right)*</i>								
First series.....	1-1-1	1-1-1	1-1-1	1-1-1	1-1-1	1-1-1	1+1+1	1+1+1
Second series.....	4+1+4	5-0-5	4+1-4	4+1-4	4+1-4	5-1-1	4+1-4	4-1+4
Third series.....	1-0-3	3-1-3	3-0-3	3-0-3	3-0-3	3-0-3	3-0-3	3-0-4
Fourth series.....	2-1-2	2-1-2	2-1-2	2-1-2	2-1-2	2-1-3	2-1-2	2-1-2
Fifth series.....	3-0-3	3-0-3	3-0-3	3-0-3	3-0-3	3-0-3	3-0-2	3-0-3
Sixth series.....	1+1+1	2-1-2	0-0-0	1+1+1	1+1+1	1+1+1	1+1+1	1+0+0
Total breast plates...	30	36	29	32	32	31	31	31
Fin rays								
First dorsal.....	5	6	4	5	4	4	4	5
Second dorsal†.....	5	6	5	5	5	5	5	5
Anal†.....	6	5	5	5	5	5	5	5
Caudal§.....	11	11	11	11	11	11	11	11
Pectorals.....	13-13	14-13	14-14	14-14	15-15	14-14	0-14	13-13

*The dashes indicate a continuous series; the + marks, an interrupted one; see text for description of series.

†Last two rays counted as one.

§Counting all articulated rays (excluding the two dorsal and the one ventral "rudimentary," non-articulated rays).

on both sides of the median scute in Variant 1, and has no median plate in Variant 2. The third series, which arches upward and backward to the scutes just before the pectoral bases, has a median scute in Variant 2 only; the modification of this row on one side of Variant 1 is noted above. The fourth series, which arches in a similar curve to the lower ends of the pectoral fins, is relatively constant, as is the fifth series, which crosses the breast in front of the pelvic fins and extends upward to behind the lower edge of the pectorals. The sixth row, which is extremely variable, is made up of small plates interpolated between the larger scutes of the preceding row. Minute spiny scutes are developed about the pelvic bases in some fish, and are numerous about the anus in all. A few small to medium-size plates are irregularly developed in several of the specimens, behind the spiny peritroct region.

FIN RAYS

The two variants show a tendency toward an increased number of dorsal and anal rays and perhaps toward a reduced number of pectoral rays (table I). Latitudinal variation may be the explanation, for the average number of dorsal and anal rays generally increases toward the north, and a reversed gradient seems to be common for the number of pectoral rays. One of the California specimens has no trace of a pectoral fin on the left side, where the position of the fin is marked by a socket-like depression; the right pectoral is normally developed.

OTHER CHARACTERS

Except as indicated above the variant specimens agree well with Jordan and Evermann's (1898, pp. 2085-2086) excellent description of *Odontopyxis trispinosus*. Detailed comparisons fail to uncover other reliable differences. The proportions are about the same.

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Atlantic Salmon Redds and Artificial Spawning Beds

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ABSTRACT

Atlantic salmon select as redd sites places where the current is accelerating. Eggs in the redd are in definite pockets associated with large gravel and stones, and are not scattered through the redd. The completed redd is figured. In Moser river, Nova Scotia, the spawning beds are poor and few. Eggs are smothered in some redds. Artificial spawning beds were prepared and were promptly used by the salmon. These provide a means of extending the area for successful natural spawning.

Although much has been written about the redds of the Atlantic salmon (*Salmo salar*), no one seems to have described their actual structure. Hobbs (1937) has done this for the quinnat salmon (*Oncorhynchus tshawytscha*), the brown trout (*Salmo trutta*), and the rainbow trout (*Salmo irideus*) in New Zealand, and his account is largely applicable to the redds of the Atlantic salmon. He gives a terminology for the parts of the redd which we have used in part.

NATURAL CONDITIONS

MAKING OF REDD

The first signs of the spawning operations are excavations in the stream bed. Many of these are "trial redds" (Hobbs 1937), which, when made by grilse or small salmon, are newly-formed depressions, only ten to twelve inches wide and one to three inches deep. (1 inch = 2.54 cm.). In Nova Scotian streams the first of these will be found about the middle of October and many others are made throughout the spawning season. Many of the trial redds are mere washings of the stream bed, but others are made deep and clean and then abandoned.

THE PIT

The salmon digs the nest pit in relatively shallow water immediately above a place where there is an acceleration in the current. The most common site is just at the head of a rapids. Others are where currents converge between two obstructions or where a current is deflected by a single obstruction. They always select places where there is a good flow of water, but in the larger streams and particularly in flood-swept streams they avoid the places where there is a very

swift current. They will make redds in a mixture of sand and fine gravel which contains little coarse gravel, but we have never found them using coarse gravel free of fine gravel and sand.

As has been noted by a number of observers (*e.g.* Belding 1934), the redd is made entirely by the female. Where the stream bottom is fairly compact, as much as a week may be required before the pit is ready for the spawn. The pit varies in size according to the size of the salmon. Female grilse or small salmon of five pounds (1 lb. = 0.45 kg.) or less in weight may make a pit not more than six to eight inches deep and about 18 inches in diameter. Those made by fish up to twenty pounds in weight may be a foot or more in depth and over three feet in diameter. Greeley (1932) has noted for brook, brown and rainbow trout that the pit is "longer than the female making it and deeper than the greatest body depth of this fish," and this is fairly true for the salmon. At the bottom of the pit is the "egg-pocket" (Hobbs 1937) in which we have invariably noted several large stones lying loosely with all the finer material washed away from them. For related species Greeley had noted large stones in the bottom of the pits, and Hobbs (1937) when opening redds, noticed the association of eggs in the egg pocket with "odd stones of more than average size." Just prior to the deposition of the eggs, the egg pocket is always the cleanest part of the pit.

SPAWNING

In the smaller tributary streams most of the adult salmon retire to the deeper pools or leave the stream during daylight. We have seen at twilight during the spawning season numbers of salmon returning from the river up a small tributary to their spawning grounds, which were a quarter of a mile (0.4 km.) from the river. They will run either up or down stream from their resting pools to their spawning grounds. In shallow spawning areas in the smaller streams, where observations on spawning can most easily be made, the spawning beds are largely deserted during daylight. Moreover, since the female spends hours or even days at a pit and the spawning act at any one pit is accomplished in a few seconds, one may spend long periods of careful observations without seeing the spawning act.

The most detailed description of the spawning of the Atlantic salmon is that given by Belding (1934). The spawning of closely related Salmonidae has been described by Greeley (1932), Needham (1934), and Smith (1941), and their observations agree very closely with those of Belding. Belding states "a female salmon was observed to spawn once in seven hours of intermittent digging." Greeley says that "a single act of spawning occurs at a single nest pit," and these observations have been confirmed by other observers.

LOCATION OF EGGS IN REDD

It seems to have been quite generally believed that the eggs of the Atlantic salmon are scattered throughout the gravel of the redd. Calderwood (1931) has stated "I have opened up a line right down a spawning ford from top to bottom. In the whole line I found eggs thinly distributed, never many together."

We have opened a considerable number of Atlantic salmon redds and we

have found only an occasional egg up in the gravel of the redd, whereas from several hundred to nearly a thousand were together in the bottom of the pit, that is in the egg-pocket. For other salmon and trout the concentration of the eggs in the egg pocket has been noted by Greeley 1932, Needham 1934, Hobbs 1937, and Smith 1941.

FERTILIZATION

Eggs and milt are shed simultaneously into the egg pocket by the female and male salmon.

We have made artificial pits with loose stones in the bottom such as those found in natural redds, and we have introduced freshly-stripped eggs and dry milt from a wide-mouth bottle by opening the bottle under water and releasing the eggs and milt just above the stones in the egg pocket. Some of the milt was immediately washed away, but it took several minutes for all the milt to clear from among the eggs and large stones. Such a deposition must closely simulate the natural deposition. Needham (1934) has noted for the steelhead trout (*Salmo gairdnerii*) that "The milt settled in a more or less compact way about the eggs though some of it was carried away by the current." It is evident that in natural spawning there is plenty of time for fertilization of the eggs before the milt is washed away, and this is amply borne out by the high fertilization of eggs in the natural redds. We have removed eggs from a new salmon redd and after placing them in a hatchery trough we have had a 78% hatch of the eggs. Hobbs (1937) made a microscopic examination of eggs from 32 redds of the brown trout and found an average fertilization of 99.2%. He states "When a total loss in a redd exceeds 3.3% the explanation should be found in causes other than initial infertility."

COVERING EGGS

It has been noted (Greeley 1932, Smith 1941) for other redd-making salmonids (excepting *Salvelinus fontinalis*, Smith 1941) that the female begins covering eggs immediately after spawning by moving the loose coarse gravel just upstream of the eggs. Such an action causes this material to fall into the egg pocket over the large stones around and under which the eggs are clustered. In opening Atlantic salmon redds we have repeatedly noticed that the cleanest material is that immediately over the egg pocket, so apparently the Atlantic salmon does the first covering similar to that of other members of the genus. After this initial covering the female works leisurely, building material over the pit. If sufficient material is not easily loosened just above the pit, she will drive it obliquely across the current towards the upper edge of the pit and in doing this forms a fan-shaped depression above the redd. Since the later materials covering the redd are freshly dug, the only parts washed away by the actions of the fish and the current are the lighter materials—silt, detritus and mud.

THE COVERED REDD

A completed redd has the form of a low mound longer than wide with the upstream face having a slope of about thirty degrees. The uppermost egg pocket is generally located beneath this face.

In the streams where we have made observations each redd generally has but a single egg pocket, but some contain two, and one long redd was found to contain four egg pockets. When an additional pit is formed it is made at the base of the upstream face of the preceding covered pit. Redds with multiple egg pockets are most often formed where plenty of fairly clean gravel is available.

Instead of being a haphazard pile of material with eggs scattered throughout it, the redd of the Atlantic salmon is a definite structure with the eggs in one or more prepared receptacles (fig. 1).

In an open pit we found that milt was only slowly washed out of the egg pocket, and Hobbs made a similar observation by using potassium permanganate crystals indicating a very slight current in the bottom of the open pit. Much fine material is used in covering the redd, and this must render the upper layers relatively impervious to water. Also while the eggs and fry are in the redd the top layers are gradually settling and at the same time accumulating bottom-shifting silt, new growths of diatoms and aquatic insect webs which catch detritus. The whole tendency is to form a more impervious layer over the redd. It would seem that this layer would be apt to cause suffocation of the eggs or alevins, yet we have

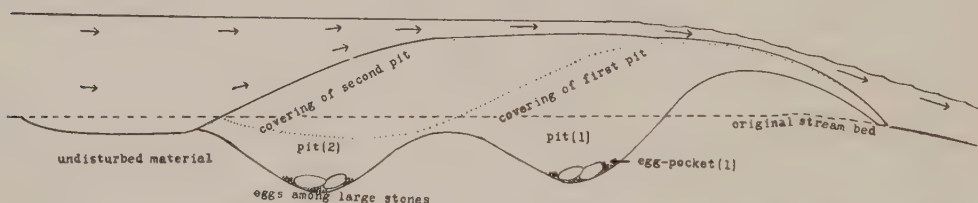


FIGURE 1. Vertical longitudinal section of salmon redd at head of rapids with two egg pockets. Arrows indicate current. Interrupted line—original stream bed; dotted line—stream bed after first pit is covered; continuous line—stream bed after second pit is covered.

opened such redds and have found hundreds of vigorous fry in the looser underlying materials. It is thus evident that there must be a considerable seepage of water through the lower looser parts. The location of the redd at a point where there is an accelerating current would tend to increase such seepage.

The newly hatched fry remain in the egg pocket or at least very closely associated with it, but fry with yolk sacs nearly absorbed may be well up in the redd. We are somewhat at a loss to know how the fully developed fry manage to escape through the compact upper layers and our observations indicate that in some redds many fry are unable to do so. In flood-swept streams the tops of the redds are generally so completely torn away by winter or spring freshets that the position of the redds cannot be recognized and such streams are most prolific of fry.

EXPERIMENTAL CONDITIONS

During the investigations on the Margaree river of inner Nova Scotia, it was found that naturally hatched fry in the Northeast branch were present in numbers in excess of those required to produce a supply of advanced parr to fully stock the stream.

The more recent investigations made on the Moser river of outer Nova Scotia reveal a very different condition. This stream has many lakes, is not flood-swept, and has few areas of gravel suitable for spawning. Over the greater part of the stream bed the gravel is firmly embedded among large stones and boulders and much of the gravel is covered with sand and organic matter. Over this bottom there is often a mat of rooted aquatic plants, consisting mainly of hornwort, *Eriocaulon septangulare*, water lobelia, *Lobelia Dortmanna*, and quillwort, *Isoetes* sp. Food for young salmon is fairly abundant and in the lower parts of the river there are few fish-eating birds, yet we found a scarcity of both salmon parr and fry.

EXTENT OF SPAWNING

It was found that the salmon made redds in all the available spawning areas of the lower four miles (1 mi. = 1.60 km.) of the river and the tributaries entering that part. In the lower mile of the river there are usually from fifty to seventy-five redds each year. In the three miles above this area a few salmon spawn in small spawning beds which are widely separated. In the vicinity of the Salmon hole about four miles up the river there are generally from ten to twenty redds. North brook, one of the larger tributaries entering the river just above the Salmon hole has the best spawning beds found and in its lower half-mile there are usually forty or more redds.

Mill brook, on which there are Mill and Second lakes, enters the river about three-quarters of a mile above tide water. This too is one of the larger tributaries. No salmon spawned in the short portion below Mill lake, and only ten to twelve redds have been found in the mile and a half of the brook between the head of Mill lake and the outlet of Second lake. A mile of stream above Second lake has the principal spawning beds and in this area there have been from twenty to forty redds yearly.

Johnny Smith brook, a small clear stream three to six feet (c. 1 to 2 m.) wide, enters Mill brook below Second lake. Although small, it has beds where about twenty redds can be made, and is generally fully utilized.

In the areas examined, redds are deficient in extent for nearly three miles below the Salmon hole on the main river and all of Mill brook below Second lake. In the other areas there is sufficient spawning for a potential supply of fry to give a good stocking of those parts, and in North brook and Johnny Smith brook the potential fry are more than enough for the stocking of those streams.

CONDITIONS OF REDDS

In the lower part of the river we found that the redds were badly silted, and when opened at the time of fry emergence they contained very few live fry, which were in or very near the egg pockets at a time when they should have been already out of the redd or at least well up in it. Large numbers of dead, partly decomposed eyed-eggs were in the egg pockets and were covered with silt and detritus.

Up the river at the Salmon hole, where the salmon had spawned in gravel containing an excessive amount of sand, eighty per cent of the eggs were dead in the eyed stage, but still in a good state of preservation, and twenty per cent

had produced fry which were still deep in the redds. The loss of eggs in this area may have been still heavier, for according to Hobbs (1937) in brown trout redds "When heavy losses occur—mortality is much higher before eyeing occurs than at any later time." It is possible that eggs which died earlier may have disintegrated.

In North brook conditions in the redds seemed to be good and the fry, although not as far advanced as those in the river, were distributed through the upper layers of the redds. There we found no smothered eggs in the redds.

Most of the redds in Mill brook below Second lake were similar to those at the Salmon hole on the main river. In Johnny Smith brook there was a fairly good emergence of fry. No smothered eggs were found, but some entombed fry were found in the redds.

ARTIFICIAL SPAWNING BEDS

Having found that there were few good spawning beds available, and that there was a loss of eggs and fry because the salmon were using inferior spawning beds, we began experimenting with prepared or artificial spawning beds.

In the fall of 1939 we prepared beds of gravel in the main river by loosening and raking the gravel back and forth in the current to remove most of the silt and dirt. These beds were made in different locations and bounded in various ways by large stones. We found that where cleaned gravel was made available above one of the stone boundaries which formed a bar across the current, the salmon made redds above the bars and utilized the cleaned gravel to cover their redds. These first beds were prepared in areas where the salmon had previously spawned.

In the following year (1940) we prepared beds of two kinds in Mill brook in places where there had been no previous spawning: (1) In about a mile of this brook the only gravel is that which is embedded among large stones on the rapids, but there are along the stream bed a number of bars of fairly clean gravel over which water flows during periods of high water. At several of these bars we built stone barriers across the brook so as to raise the water level and cause part of the flow of the stream to pass over the gravel. When the salmon began spawning they made redds on the flooded gravel bars. No further modification or repair of these bars was made, and in the fall of 1941 the salmon spawned again in these beds. We have no assessment of the value of these, excepting that during the summer of 1941 moderate numbers of salmon fry were found adjacent to these areas, whereas in previous years none were found. (2) In the lower part of Johnny Smith brook and in Mill brook below Mill lake the embedded stones were dislodged from the gravel, and straight bars were constructed across the stream. When the embedded stones were removed, the gravel was then available to the salmon and they made redds on the upstream side of the bars but only one or two redds were made at each.

V-SHAPED BARS

It was observed that the salmon made their redds above the straight bars only where the currents tended to converge and accelerate. The digging of the

pit was started just above the point of maximum convergence of the currents. With this observation as a basis, we made in the fall of 1941 a number of small beds, each of which provided what was requisite for making a single redd. These were on a stretch of Mill brook below Second lake, where the bed of the rapids is composed of large stones and gravel, and where no salmon had previously spawned. The stones, some of them about a foot (30 cm.) in diameter, were so solidly embedded that a pick was required to loosen them.

To make one of these beds we selected a place where the water at normal height was from six to ten inches (1 inch = 2.54 cm.) deep and there we cleared the large stones from a V-shaped area about four or five feet wide at the base, that is the upstream end, and about five feet long. The stones removed from the interior were used to make low bars along each side of the V. At the apex of the area, that is the downstream end, we embedded one of the larger stones so that it

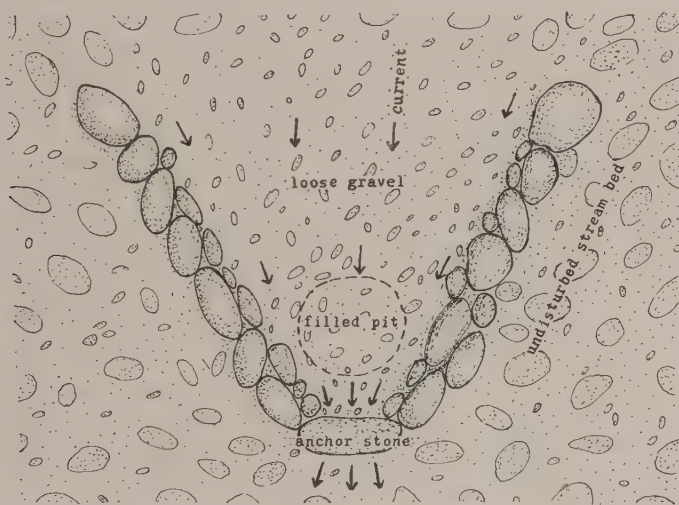


FIGURE 2. An artificial spawning bed for salmon within a low V-shaped bar of stones.

was lower than the bars and acted as an anchor over which there was a strong flow of water. The gravel within the area was then loosened with a pick to a depth of three or four inches and was raked back and forth to free it from detritus and silt. About eighteen inches above the apex where the currents converged we made a pit about eighteen inches in diameter and ten inches deep, and filled it with loose gravel. During the construction, the water inside the area was deepened by removal of the large stones and the raising of the water level by the bars. The completed structure (fig. 2) consists of a V-shaped area, with a bar on each side six to ten inches high, an embedded anchor rock at the apex over which the converging currents, shown by arrows, flow. Inside the bars there is a layer of comparatively clean loose gravel with the filled pit above the apex.

On October 21, 1941, when the salmon had already begun spawning, four of these were constructed in close proximity, and on October 22 each contained a completed redd. The salmon had opened the pits, throwing the gravel out over

the anchor stones, and after depositing eggs had covered the pit with the loose gravel readily available inside the bar.

In all, twelve of these experimental beds were constructed over a period of three days. Each bed was occupied by salmon the first night and the redd was completed within twenty-four hours.

This type of bed has the following advantages: It is easily constructed. It can be made in places where small amounts of material are available, or, if materials are plentiful, as many as may be desired can be constructed. It is readily accepted by salmon, and they can easily construct their redds in it. Since the bottom currents converge toward the position of the egg pocket, all the prepared gravel within the bars can be used for covering the redds.

EFFECTIVENESS OF ARTIFICIAL BEDS

We have made no definite assessment of the emergence of fry from the artificial beds. We attempted to enumerate the fry just before emergence by placing a long net of fine wire screen below the redd while it was being opened, but we found that the fry tended to swim against the current or seek shelter among the stones rather than drift with the current into the net. Only a part of them could be recovered. However, it was determined that where the redds were made of pre-cleaned gravel there was very little silting of the redd, and consequently no smothering of the eggs such as we had found in many of the natural redds. Advanced fry were well up in the redd material and not entombed in the egg pocket. The redds were seemingly equal to those made in good natural spawning beds.

APPLICATION

In many salmon streams there are extensive areas which are little or not at all utilized as rearing grounds for young salmon because they lack or have inferior or scanty spawning grounds. Where the current is suitable and the necessary materials are readily available, artificial beds could be easily constructed to provide through natural spawning a satisfactory supply of fry for such areas.

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Glucose Metabolism of Bacteria from Commercial Fish

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ABSTRACT

The products of fermentation of glucose by "resting cell" suspensions of certain bacteria (*Serratia*, *Achromobacter*, and *Micrococcus*) isolated from decomposing cod muscle include lactic acid, acetic acid, formic acid, ethyl alcohol, carbon dioxide and small amounts of acetylmethylcarbinol. With increased acidity in the fermentation system there is a marked increase in the percentage of lactic acid formed, with a corresponding decrease in the other products. The optimum pH for the fermentation of glucose appears to be in the vicinity of 6.8—that is at, or near, the pH of fresh cod muscle.

INTRODUCTION

In 1939 Beatty and Collins reported investigations on "the breakdown of carbohydrates, proteins and amino acids during the spoilage of cod muscle press juice" and showed that this breakdown was almost exclusively due to bacterial activity. According to these workers the early decomposition of cod fish muscle occurs essentially in two stages. The first of these is the breakdown of carbohydrates and carbohydrate derivatives, especially lactic acid, with the simultaneous reduction of trimethylamine oxide to trimethylamine. The second phase involves the degradation of proteins. This latter stage apparently does not begin until most of the lactic acid and fermentable carbohydrates have been consumed.

The results were obtained with cod muscle press juice contaminated with the normal heterogeneous flora of fresh fish. It seemed desirable to study the metabolic products evolved during the fermentation of glucose by pure cultures of the bacteria most commonly associated with the first stage in decomposition of cod muscle.

EXPERIMENTAL

ISOLATION OF CULTURES

A large number of colonies were picked from nutrient agar plates poured as dilutions from decomposing cod muscle and cod muscle press juice. Since the trimethylamine producing organisms may be considered most significant during the first stage in decomposition, such were selected for this study namely *Serratia marcescens* (cultures 5J29, 59), *Micrococcus candidus* (cultures 9, 5J36), *M. aurantiacus* (culture 16), *Achromobacter* sp. (culture 5J21), and an unidentified Gram positive rod.

RESTING CELL SUSPENSIONS

In order to simplify the analytical work and to reduce variations attendant with the use of growing cells, non-proliferating or "resting cells" (Quastel and Whetham 1924) were employed. The majority of the cultures grew profusely on agar plates having the following composition: Tryptone, 0.5%; yeast extract (Difco), 0.5%; glucose, 0.1%; trimethylamine oxide, 0.1%; agar, 1.5%.

The organisms were transferred from stock agar slopes to broth having the same composition as the agar described above. These cultures were incubated at 25°C. for 24 hours, at the end of which Petri dishes were inoculated and spread with 1 ml. of the culture. After 24 hours' growth on the plates, the cells were washed off with distilled water, centrifugalized, resuspended in distilled water, and centrifugalized again. Finally they were suspended in an M/7.5 phosphate buffer. Experiments showed that distilled water was just as satisfactory as physiological salt solutions or buffer solutions for cell washing.

ANALYTICAL METHODS

Trimethylamine was determined by the method of Conway and Byrne (1933) as applied to trimethylamine by Beatty and Gibbons (1937).

The copper-iodometric method of Schaeffer and Somogyi (1933) was employed for the estimation of *glucose*, the cells and proteins, when present, being removed by the mercuric sulphate-barium carbonate precipitation of West, Scharles and Peterson (1929).

Lactic acid was determined by the method of Friedemann and Graesser (1933) after removal of interfering substances by precipitation with copper sulphate and calcium hydroxide.

Volatile acids, with the exception of formic, were determined by the method of Friedemann (1938). For the determination of formic acid a greater accuracy was attained by employing the much more specific mercuric chloride reduction method (Assoc. Off. Agric. Chem. 1940 p. 466). Van Niel (1928, p. 94) and McNair (1933) have noted a slight production of formic acid on distillation of solutions containing glucose and lactic acid in the presence of sulphuric acid. Since test distillations showed that the amounts of formic acid produced in this way were extremely small, no correction in the analytical values was found necessary. The only other volatile acid found was acetic acid.

Ethyl alcohol was determined by the method of Friedemann (1938).

For the estimation of *carbon dioxide* Van Slyke's manometric method was employed (Hawk and Bergeim 1938, pp. 499-503). When the cell suspension system was very heavily buffered, 0.5 N sulphuric acid was substituted for lactic acid to ensure complete liberation of the dissolved carbon dioxide.

Acetylmethylcarbinol and *diacetyl* were estimated by the method of van Niel (1927). These two substances were found to be responsible for the slightly positive results in testing for 2, 3-butylene glycol (Brockman and Werkman 1933).

pH AND PRODUCTION OF LACTIC ACID

Preliminary experiments indicated that the proportionate yields of the fermentation products were to some extent dependent upon the hydrogen-ion concentration of the medium. The effect of the initial hydrogen-ion concentration was therefore studied using the following system:

Glucose solution (18%).....1 ml.
 Trimethylamine oxide (22%).....1 ml.
 Cell suspension of culture 59 in distilled water.....3 ml.
 Phosphate buffers (M/7.5) of various hydrogen-ion concentrations to a volume of 50 ml. in volumetric flasks.

The flasks were incubated at 35°C. Samples were withdrawn after two and five hours for the determination of glucose and lactic acid.

The results (table I) demonstrate that the more acid the fermentation system the higher is the yield of lactic acid. Despite the comparatively heavy buffering of the medium the reaction shifted towards the acid side as the fermentation proceeded. This change in the hydrogen-ion concentration appears to be the reason for the higher yields of lactic acid after five hours than after two hours, since other experiments in which the pH was kept constant have shown that time does not change the ratio of the products materially. Similar results were obtained using several other cultures, the general effect being that the more acid the system the higher the yield of lactic acid. Friedemann and Kmiecik (1941) working with pathogenic *Clostridia* have observed that the yield of lactic acid "was greatest in the medium in which the greatest consumption of sugar was found." Smith and Sherman (1941) found the percentage of lactic acid formed from glucose by certain *Streptococci* to be quite variable from culture to culture. The relation of the present results to those already reported in the literature is not immediately evident. They are given here to show the significance of controlled pH in the experiments which follow.

TABLE I. Effect of hydrogen-ion concentration on lactic acid production.

Initial pH	pH after 5 hours	Yield of lactic acid as per cent of glucose	
		2 hours	5 hours
4.6	4.4	91.6	95.2
5.4	4.9	66.6	80.6
5.9	5.3	59.3	74.8
7.1	6.3	28.9	40.9
8.1	7.2	21.7	29.9

pH AND RATE OF FERMENTATION

The experimental procedure was the same as previously recorded. The relation of the initial hydrogen-ion concentration to the amount of glucose used by representative cultures was determined (figure 1). The optimum reaction

for the fermentation of glucose by these cultures appears to be in the vicinity of pH 6.8. All cultures were more sensitive to acid conditions than to alkaline. The results confirm the conclusions of Nadeau (1940a, 1940b) regarding acid dips as fish preservatives. They are also in agreement with those of Cook and Alcock (1931) who demonstrated that in the case of *B. coli* the pH-activity curve rises steadily from an acid reaction to a plateau beginning at pH 8.0 or higher depending upon the substrate.

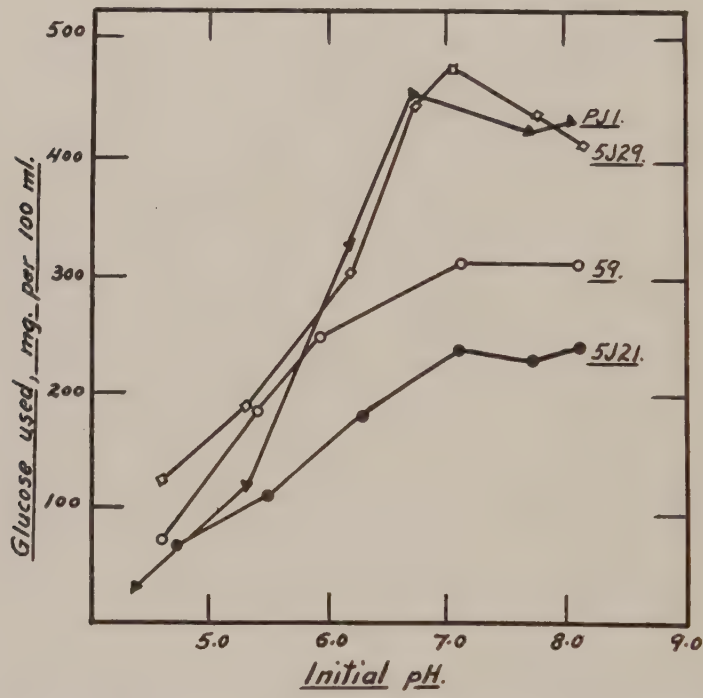


FIGURE 1. Effect of pH on amount of glucose used in 3 hours by four representative cultures at 35°C.

PRODUCTS OF FERMENTATION

To determine the products of fermentation of glucose the following system was used:

- Glucose.....0.9 g. per 100 ml.
- Trimethylamine oxide.....1.1 g. per 100 ml.
- Cell suspension in M/7.5 phosphate buffer at pH 7.2 to a volume of 300 ml.

The fermentation systems were prepared in 500 ml. Erlenmeyer flasks stoppered with rubber stoppers and incubated for 24 hours at 35°C. In the case of very active cultures the concentrations of glucose and trimethylamine oxide were doubled and the strength of the buffer was increased. This did not affect

the results as long as the hydrogen-ion concentration remained fairly constant. The results given (table II) are from duplicate determinations. Quantities are expressed as millimoles per 100 ml. and in percentages of the glucose accounted for by each of the products.

The results of table II for cultures 59 and PJ1 at pH 7.5 and 5.5 substantiate those previously obtained and shown in table I regarding the effect of pH on the products of fermentation. They show that at the lower pH there is an increase in the yield of lactic acid and a corresponding decrease in the yield of the other products, the total recovery in both cases being approximately the same.

In spite of the heavy buffer employed the hydrogen-ion concentration was subject to some changes during the course of the fermentation. The results for culture 5J21 illustrate the effect of these variations. The initial pH (7.2) was the same in both experiments. In the first only 1.6 mM per 100 ml. of glucose was dissimilated. The pH had fallen to 7.0 at the end of the incubation period. In the second run 4.9 mM per 100 ml. was fermented, and in this case the pH had fallen to 5.9. As a result the yield of lactic acid was increased while a corresponding increase in the other products occurred through the shift in the reaction. The relative proportions of the products were thus influenced by the amount of substrate dissimilated, which in turn was dependent upon the concentration of the cell suspension and the length of the incubation period.

The first set of values given for culture 59 at pH 7.2 at first sight appears contradictory to the others given for the same culture. The yield of the lactic acid is much greater than could have been expected at this pH. The amount of glucose dissimilated, however, is much greater than in the other two sets of values given. This increased utilization of glucose, with consequent increase in the total amount of lactic acid formed, lowered the pH so that in the latter period of fermentation the reaction was occurring at a pH which favours the formation of larger amounts of lactic acid (per mole of sugar decomposed). The high recovery of the glucose carbon in this particular set was not attained in subsequent experiments but the data have been included to indicate the extent of variation which may be anticipated. A more thorough standardization of the technique made it possible to obtain repeatable recoveries of the total glucose on repetition of the experiments, as demonstrated by the other two sets of values given.

The results do not furnish very definite indications as to the mechanism of fermentation, and in some cases there does not appear to be any clearly explainable relationship between the relative amounts of the products. In other cases, however, such a relationship is evident. Thus culture PJ1 produces almost exactly equimolecular amounts of acetic acid and formic acid, as well as of ethyl alcohol and carbon dioxide. The results for culture 5J29 show that, if the molar yields of acetic acid and ethanol are added, and the sum is compared with the sum of the molar yields of formic and carbon dioxide, the 2-carbon derivatives occur in equimolecular amounts to the 1-carbon derivatives. Both cases were suggestive of a 3-carbon intermediate. It would appear that this intermediate could not be lactic acid, since other experiments have shown that lactic acid does not give rise to all these products under the conditions of these experiments and with the

same cultures. Furthermore lactic acid fermentation by the organisms under these conditions is much too slow to permit its consideration as an intermediate in these reactions.

By way of comparison it may be noted that only cultures 59 and PJ1 do not produce carbon dioxide in appreciable quantities. Aside from this, there is a general similarity in the products, although the proportions vary somewhat. The value listed for acetylmethylcarbinol include traces of diacetyl, since the van Niel (1927) distillation without the ferric chloride gave positive results but a smaller crop of crystals.

TABLE II. Products of fermentation of glucose by various cultures. Temperature 35°C.

Culture init. pH	Unit of measure- ment	Glucose used	Lactic acid	Acetic acid	Formic acid	Ethyl alcohol	Carbon dioxide	Acetyl methyl carbinol	Total recovery of glucose carbon
<u>59</u>									
pH 7.2	mM	2.46	3.78	0.67	0.72	0.64	0.0		
	%		76.9	9.07	4.89	8.66	0.0		99.5
pH 7.5	mM	1.36	1.08	0.73	0.78	0.36	0.0		
	%		39.7	17.9	9.7	9.30	0.0		76.6
pH 5.5	mM	0.74	0.87	0.13	0.12	0.19	0.0		
	%		58.9	5.85	2.72	8.55	0.0		76.0
<u>PJ1</u>									
pH 7.5	mM	1.35	0.70	1.24	0.52	0.62	0.0	0.03	
	%		26.0	30.6	19.1	15.3	0.0	1.5	92.5
pH 5.5	mM	1.86	2.77	0.45	1.10	0.26	0.0	0.04	
	%		74.5	8.06	9.85	4.66	0.0	1.4	98.5
<u>5J21</u>									
pH 7.2	mM	4.90	3.55	2.10	2.14	0.60	0.59	0.18	
(final* 5.9)	%		36.3	14.5	7.10	4.10	2.0	2.5	66.5
pH 7.2	mM	1.60	0.70	1.06	1.02	0.33	0.35	0.05	
(final* 7.0)	%		21.9	21.9	12.5	6.2	3.7	2.1	68.3
<u>9</u>									
pH 7.2	mM	1.83	1.45	1.38	0.29	0.31	0.60		
	%		39.6	25.2	2.63	5.65	5.45	positive	78.5
<u>5J29</u>									
pH 7.2	mM	8.86	7.74	4.53	3.04	1.18	2.63		
	%		43.7	17.0	5.74	4.43	4.95	positive	75.8
<u>PJ36</u>									
pH 7.2	mM	0.22	0.1	0.1	0.20		0.48		
	%		23.0	15.4	15.4		31.0	positive	84.8
<u>16</u>									
pH 7.2	mM	4.50	2.90	2.17	1.24	1.33	3.08		
	%		32.2	16.0	4.6	9.85	11.4	positive	74.0

*pH at the time of analysis.

The values for trimethylamine are not listed in table II, but in general the cultures would reduce between one and two millimoles of the trimethylamine oxide for every millimole of glucose fermented.

The fate of the glucose not accounted for remains unknown. Tests were made for succinic acid by the method of Goepfert (1940) but only small quantities were ever found to occur. Only very slight amounts of pyruvic acid could be detected (Case 1932). No tests were made for glycerol and it is not impossible that it may have occurred in measurable amounts.

DISCUSSION

In considering these results it may be noted that the experiments have been carried out under a certain definite set of conditions, and that there is evidence to show that by changing these conditions very considerable quantitative changes may be brought about in the results. The effect of hydrogen-ion concentration has already been discussed but other variables are also significant. In a fermentation system prepared as was done in these experiments, the conditions will be semi-anaerobic, aerobiosis taking place mainly at the surface and anaerobiosis in the interior (Watson 1939). This of course is similar to conditions actually prevailing on the surface and in the interior of spoiling fish muscle. Preliminary experiments have shown that strictly aerobic conditions on the one hand, and strictly anaerobic conditions on the other, will lead to quite a marked difference in the fermentation products.

The presence of protein may affect the results to a certain extent. Certain of the cultures studied fermented glucose very slowly, but with some of these the fermentation could be stimulated markedly by the addition of peptone to the system. As yet no investigations have been undertaken to determine how the peptone affects the stimulation, but a similar influence has been noted on the rate of fermentation by *Streptococcus lactis* (Rahn, Hegarty and Deuel 1938).

One could then conceive of the following series of reactions as taking place: glycogen gives rise to glucose which is broken down by bacteria to lactic acid and the other products of fermentation listed in this paper. The lactic acid is then further dissimilated along with the preformed lactic acid in the muscle.

The time relationships for these reactions are not easy to determine, since lactic acid may both be formed and utilized at the same time, while the formation and dissimilation of glucose is occurring simultaneously. The dissimilation of the sugar however, proceeds in preference to that of lactic acid since (1) lactic acid does increase in muscle at the beginning of storage (Beatty and Collins 1939) and (2) the rate of breakdown of glucose by pure cultures proceeds at a more rapid rate than that of the lactic acid.

SUMMARY

Studies on the degradation of glucose by certain bacteria (*Serratia*, *Achromobacter*, *Micrococcus*) isolated from cod muscle have revealed that a variety of factors influence the rate and path of fermentation. It has been found that the hy-

drogen-ion concentration in the fermentation system markedly affects the dissimilation products, in general the more acid the system the higher the yield of lactic acid. The products of fermentation have been determined quantitatively and include lactic acid, acetic acid, formic acid, ethyl alcohol, carbon dioxide, and small quantities of acetylmethylcarbinol.

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The Surface Concept in Measurement of Fish Spoilage

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ABSTRACT

The contact plate method as used for cod muscle has revealed that the major changes rendering fish unfit for human consumption can be attributed almost entirely to surface pollution of the fish with spoilage bacteria. This is confirmed by three chemical tests, trimethylamine, tyrosine, and surface pH. The relative rates of increase in all three are much greater at the surface than in the interior of cod and haddock fillets. The more rapid surface changes are taken as evidence that tests for spoilage in fish products should be based on samples from the surface of the products and not from composite samples.

The imperative need for a satisfactory objective test for the determination of spoilage in fish and meat products is evidenced by the large number of methods which have been published (Weaver 1927, Tauti 1931, Yamamura 1932, Stansby and Lemon 1933, Kimura and Kumakura 1934, Tanikawa 1935, Boury and Schvinte 1935, Beatty and Gibbons 1937, Tarr 1941). A survey of the various procedures which have been described for fish and fisheries products shows that in most cases the sample used for the spoilage determination has been selected and prepared following prescribed analytical technique, and considerable pains have been taken to obtain a truly representative sample of the entire product. Such a method of sampling presupposes that the spoilage change under examination has occurred at points or in areas which are indefinable and thus the changes could only be measured by a composite sampling procedure. The evidence to be presented demonstrates that in the deterioration of fish or fish fillets the location of the change can be defined, the change taking place sooner and at a much greater rate at the surface than in the interior.

The work of Beatty and Gibbons (1937), Beatty and Collins (1939), Tarr (1939), and Watson (1939) has demonstrated that ordinary fish spoilage can be attributed almost exclusively to the activity of bacteria. It is to be anticipated that, at the outset at least, these bacteria will be on, or very near, the surface of the fish. This probability is supported by the results of Stewart (1930) which showed that sterile muscle samples can readily be obtained from the interior of fresh fish even when the surface is heavily contaminated with bacteria. Previously, Gee (1927, 1930) had obtained less conclusive results on the sterility of fish muscle. Gee's procedure has been criticized by Stewart as being unsatisfactory.

If it can be shown that bacteria do not penetrate to the interior, and that their effect, as measured by chemical changes, is limited to the surface, the importance of the surface concept is established.

METHODS USED

BACTERIOLOGICAL

Since bacteria are responsible for the main spoilage changes, it would be desirable to ascertain the numbers of bacteria on the surface and in the interior of fish or fish fillets. An accurate quantitative count is difficult to obtain, but the relative numbers can be satisfactorily estimated by a modification of the contact plate method proposed by Walter and Hucker (1941).

For this purpose agar plates having the following composition were prepared:

Peptic-tryptic digest of cod muscle.....	1,000 ml.
(Total nitrogen 0.5%)	
Glucose.....	5 g.
Potassium dihydrogen phosphate.....	5 g.
Agar.....	15 g.
pH adjusted to 7.2 before autoclaving.	

Precautions were taken to avoid condensation of excess moisture on the surface of the cooled plates. The contacts with the fish were made by momentarily pressing the surface of the agar to the fish or fillet. The samples for the interior contact plates were prepared by splitting the fish or fillet with a sterile scalpel. Particular care had to be exercised in preparing representative samples of the interior, since surface pollution is very easily carried into the interior with the passage of the scalpel. After a short practice satisfactory contacts could be obtained. The various plates were incubated at 22°C. for two days and were then examined and photographed.

CHEMICAL

The majority of the chemical tests for spoilage are based on the determination of the products of bacterial metabolism and to a much lesser extent on the results of autolysis. In the preliminary changes which render fish unacceptable to the consumer the bacterial changes are the dominant ones. On the basis of this, one must seek the more marked chemical alterations in the vicinity of the focal points of bacterial growth, that is, on, or very near the surface of the fish or fillet. In an effort to demonstrate this surface alteration three tests were selected, the trimethylamine determination of Beatty and Gibbons (1937), the tyrosine method of Bradley and Bailey (1940), and the change in the hydrogen-ion concentration.

The method of Bradley and Bailey (1940) for the determination of the tyrosine value was modified with a view to shortening the time required for the test. The procedure used was as follows:

Two g. of finely minced muscle were treated with 40 ml. of 5% trichloroacetic acid (Analar Reagent quality) for 15 minutes, then filtered. An aliquot of the filtrate (the volume depending on the tyrosine value) was diluted to 5 ml., 10 ml.

of 0.5 N sodium hydroxide was added and 3 ml. of the Folin and Ciocalteau (1929) reagent (dilution 1:3) was added, and the tube was shaken immediately. The colour development was measured at five minutes by means of the Evelyn Photo-electric Colorimeter, using a filter giving maximum transmission at 660 mμ.

The total time required for the test using this procedure is approximately one-half hour or less as contrasted with somewhat more than four hours using Bradley and Bailey's method. Numerous tests have indicated that reliable and reproducible results could be obtained by this modification.

The hydrogen-ion concentration was measured with the Beckman pH meter, using the glass electrode.

At the outset, samples of the fish were stirred with twice their weight of water and the resulting suspension used for the pH determination. Later it was found that the pH value could be obtained with satisfactory accuracy by imbedding the electrodes directly in the muscle of the fish or fillet. The pH values given in the data were obtained in this manner.

EXPERIMENTAL.

EXPERIMENT I

In order to obtain an estimate of the relative numbers of bacteria on the inside and on the surface of the fillets a series of contact plates were prepared from the interior and exterior respectively. Figure 1 shows the bacterial growth on plates prepared from fillets after various times of storage.

From the photographs it is obvious that the surface plates were too crowded to permit counting, even when prepared from fresh fillets. The plates from the interior contacts showed very few colonies, and there was not appreciable increase in numbers until after the surface of the fish had become spoiled. In more than 90 per cent of the contact plates prepared during the course of this investigation no colonies appeared from the interior samples.

EXPERIMENT II

Cod fillets were prepared from fish in rigor, wrapped in brown waxed paper and stored at 3 different temperatures. One or two fillets were removed, at various time intervals, examined organoleptically and sampled for analysis. The surface samples were obtained from the lateral surfaces to a depth of 1 to 3 mm. with the aid of a sharp scalpel. Analysis demonstrated that fairly uniform results could be obtained. The interior samples were taken by cutting the fillet in half transversely and removing 5 to 10 g. from the central portion.

In the results (table I) the data for the fillets stored at 0°C. show that spoilage, as detectable by any of the three chemical methods, has not yet begun after seven days. There is a steady but slight increase in the tyrosine values of the interior samples at this temperature. The absence of bacteria, in any appreciable numbers, points to the conclusion that this increase may be attributed to autolytic phenomena. Other data have, in certain cases, shown similar results, but further work is necessary for the adequate elucidation of the causes leading to this change. The increase in the hydrogen-ion concentration during the preliminary period of storage, followed by a steady decrease, has been found



FIGURE 1. Photographs of contact plates prepared from the surface and the interior of fish fillets stored at 4°C.

No. 5 and 6,—first day of storage, fish fresh.

No. 7 and 8,—second day of storage, fish fresh.

No. 11 and 12,—fifth day of storage, fish distinctly spoiled.

No. 13 and 14,—seventh day of storage, fish badly spoiled.

TABLE I. The surface and interior pH, trimethylamine and tyrosine values of fresh cod fillets stored at 0°, 5° and 10°C.

Time of storage (days)	Test	Temperature of storage					
		0°C.		5°C.		10°C.	
		Surface	Interior	Surface	Interior	Surface	Interior
1	Trimeth....	2.5	2.5	2.5	2.5	2.5	2.5
	Tyrosine...	7.6	6.4	8.3	8.5	7.2	7.8
	pH.....	6.6	6.8	6.6	6.5	6.7	6.8
3	Trimeth....	2.5	2.5	7.5	3.7	22.3	7.5
	Tyrosine...	5.7	8.3	15.9	8.8	21.4	11.0
	pH.....	6.6	6.6	6.4	6.4	6.6	6.6
5	Trimeth....	2.5	2.5	32.5	7.5	69.0	22.5
	Tyrosine...	5.9	10.1	16.5	10.0	24.1	14.4
	pH.....	6.4	6.4	7.0	6.4	7.3	6.4
7	Trimeth....	2.5	2.5	80.0	22.5	95.0	80.0
	Tyrosine...	7.1	11.6	33.1	15.6	48.7	26.0
	pH.....	6.5	6.5	7.4	6.6	7.4	7.1

to be characteristic of all cod and haddock fillets examined. The reason for this initial shift in reaction is not immediately evident, but it may be a manifestation of the changes noted by Stansby and Lemon (1933) in their potentiometric titrations of cod and haddock muscle. There was no change in the trimethylamine content. The bacterial count had apparently not reached the level which would initiate reduction of the trimethylamine oxide.

The data from the samples stored at 5°C. and 10°C. bring out clearly the relationship between the surface and interior. At all time intervals after the first day of storage the surface values are much higher than the corresponding interior values. All three chemical tests show almost complete agreement. The surface trimethylamine content increases at a greater rate than the tyrosine and pH values. This relationship with respect to pH is to be expected, since the buffering capacity of the muscle (Collins, Kuchel and Beatty 1941) against the increased trimethylamine is sufficient to prevent an immediate change in the pH. The more rapid increase of trimethylamine confirms the superiority of this test as compared to the tyrosine value.

Beatty and Gibbons (1937) have suggested that spoilage odours become apparent after an increase of approximately 4 to 6 mg. of volatile basic nitrogen per 100 g. of tissue. This conclusion was reached when a representative composite sample of the entire fillet was used for analysis. The present data show that the surface trimethylamine increases and goes beyond this odour threshold long before a gross analysis would show a corresponding rise in the volatile base. In the same way the tyrosine and pH values rise quite rapidly at the surface without appreciable change in the interior. The surface analyses of the fillets

stored at 5°C. for five days indicate that the fish has reached, or is rapidly reaching, the spoiled state. The analytical values for the interior of the same fillets would indicate that the fish is completely fresh. This conclusion is in agreement with organoleptic observations.

The data presented in table I are from surface and interior samples only, and emphasize the importance of the surface concept. To make a just comparison with the results of other workers, it is necessary to compare the degree of spoilage on the surface, in the interior, and of a composite sample of the same fillet.

EXPERIMENT III

For this experiment cod fillets were stored at 5°C. and examined at various intervals. For sampling two cross-sectional pieces about one and one-half inches (3.8 cm.) in length were cut from the thicker end of the fillet. From one of these interior and surface samples were taken as before, while the other was comminuted and mixed thoroughly for the composite sample.

TABLE II. The surface, interior and composite pH, trimethylamine and tyrosine values of cod fillets stored at 5°C.

Time (days)	pH			Trimethylamine			Tyrosine		
				(mg./100 g.)			(mg./100 g.)		
	Surface	Com- posite	Interior	Surface	Com- posite	Interior	Surface	Com- posite	Interior
2	6.7	6.6	6.5	10.0	6.0	2.0	16.0	10.1	12.4
3	7.3	7.0	6.7	46.7	21.8	7.2	17.6	11.4	8.8
4	7.7	7.3	6.9	63.0	33.5	15.0	26.0	17.6	14.1
5	7.9	7.4	7.1	72.0	45.0	30.0	29.4	23.0	19.0
6	7.9	7.4	7.1	76.5	68.0	60.2	32.0	27.5	23.9
7	8.0	7.7	7.1	80.0	81.0	79.5	33.4	30.2	27.0
9	8.4	8.0	7.5	84.5	83.0	82.5			

The curve (fig. 2) for the trimethylamine values (table II) of the composite sample is in close agreement with the result of Beatty and Gibbons (1937). From the curves in figure 3, plotted from the data in figure 2, it is to be noted that the rate of trimethylamine production reaches a maximum in 2.5 days, at the surface, at 4.75 days in the composite, and at 5.75 days in the interior. These values can only be treated as comparative, since a difference in the previous history of the fish, both with respect to time and the nature of the bacterial contamination, may considerably alter the quantitative values. Several experiments have demonstrated that the qualitative picture remains unchanged.

It is of interest to note that the trimethylamine content of the interior rises sharply after the surface has become spoiled. This is significant since bacterial contamination of the interior, commensurate with this rapid trimethylamine

production, does not occur. Figure 4 shows contact plates prepared from the fish analysed on the fourth day of storage (table II) where the surface trimethylamine nitrogen is 63 mg, the composite 33.5 mg. and the interior 15.0 mg. per 100 gm. of muscle. Despite the rise of trimethylamine in the interior, the plate prepared from this section of the fillet is still free from bacteria. From this one must conclude that this trimethylamine reaches the interior through the agency of diffusion. The cross-section contact plate (fig. 4) demonstrates strikingly the narrow zone of bacteria on the surface of the fillet and their absence in the interior. The band of growth appearing on the plate is probably thicker than

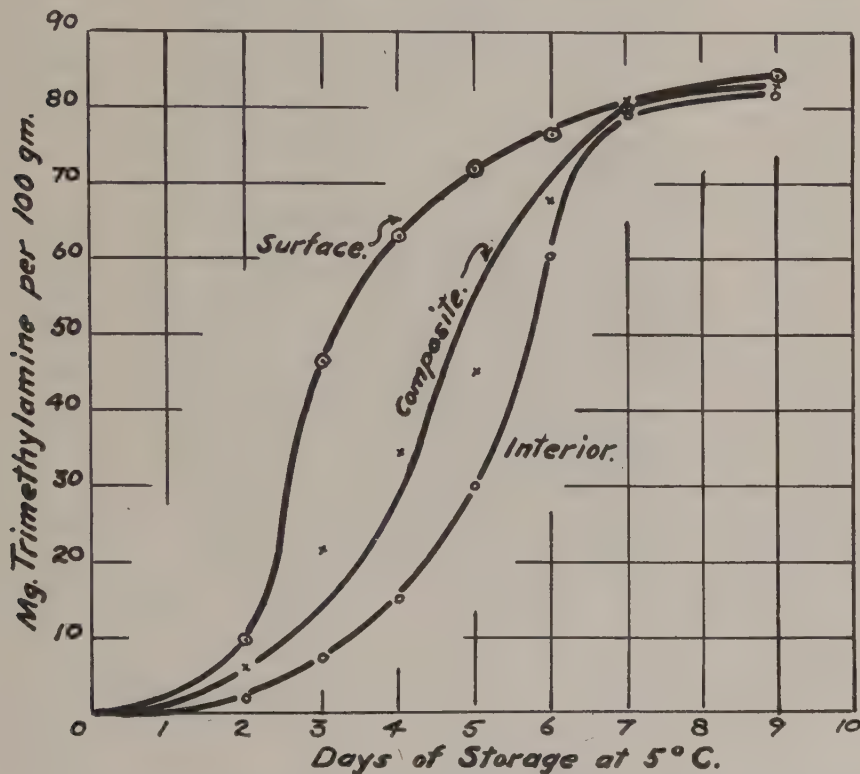


FIGURE 2. Changes in trimethylamine of surface, composite, and interior samples of fillets stored at 5°C. Trimethylamine expressed as mg. of nitrogen per 100 g. of muscle.

the zone actually occurring on the fish, since it is very difficult to cut a cross-section without carrying some of the bacteria into the muscle with the scalpel.

The tyrosine values (table II) are in essential agreement with those for trimethylamine. It is worthy of note that the three values for surface, composite and interior tyrosine content do not all reach the same point, as do those for trimethylamine.

The surface pH shows a steady rise, parallel to the increases of the trimethylamine and tyrosine. The pH value of the interior does increase with time but it lags considerably behind the trimethylamine value. For example, after 7

days of storage the amount of trimethylamine in the surface and interior are essentially equal, while there is a difference of approximately one unit in the pH values (pH 8.0 for the surface and pH 7.1 for the interior). From these results one must conclude that, in the absence of heavy bacterial contamination in the interior, the buffering capacity against base is retained. Other experiments (unpublished results) have shown that the lactic acid normally present in the muscle disappears more rapidly from the surface than from the centre. This may afford in part an explanation of the slow rise in pH despite the accumulation of trimethylamine.

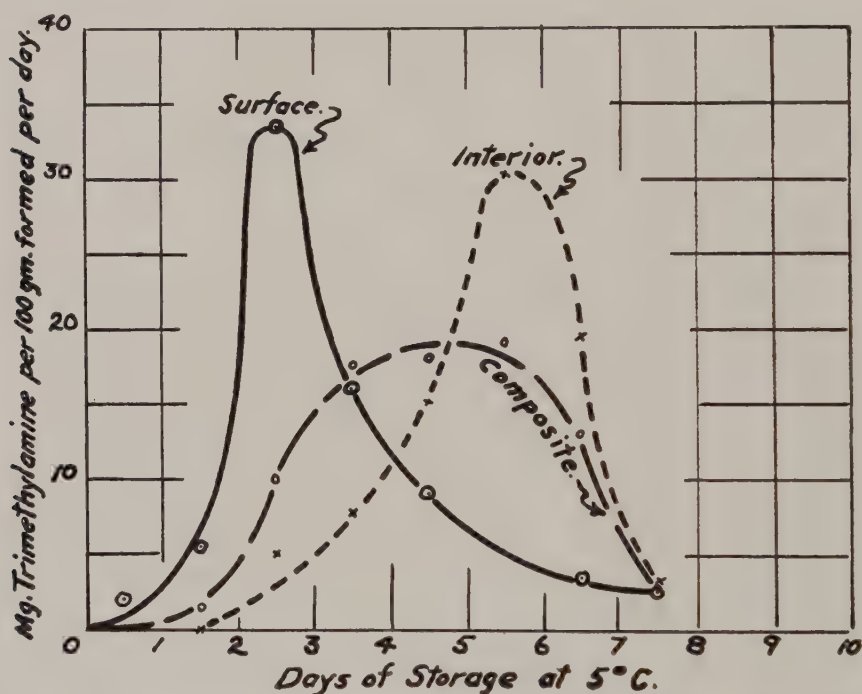


FIGURE 3. Rate of trimethylamine appearance in surface, composite, and interior samples of fillets stored at 5°C., plotted as mgs. of trimethylamine nitrogen per 100 g. per day.

DISCUSSION

The data presented prove beyond doubt that the natural spoilage rendering fish fillets unfit for human consumption occurs primarily at, or very near, the surface of the product. The results of all the chemical and bacteriological determinations applied agree completely in this respect.

On this basis it becomes evident that any tests designed to detect incipient spoilage should be applied to samples removed from the surface and not to composite ones such as ordinarily are prepared for this purpose. The focal area of bacterial contamination is actually a relatively narrow zone on the surface, and since most of the proposed tests for spoilage depend upon the end products of bacterial metabolism, these products will occur in the highest concentration at the surface.

While the present work has not included a study of meat products as such, practical and theoretical consideration would seem to justify the application of the surface concept of spoilage to such materials.

The present work has dealt with the surface concept of spoilage in relation to the measurement of changes rendering fish unfit for consumption as food. The other aspects of such a concept in relation to proper processing and preservative treatment of fisheries products are evident.

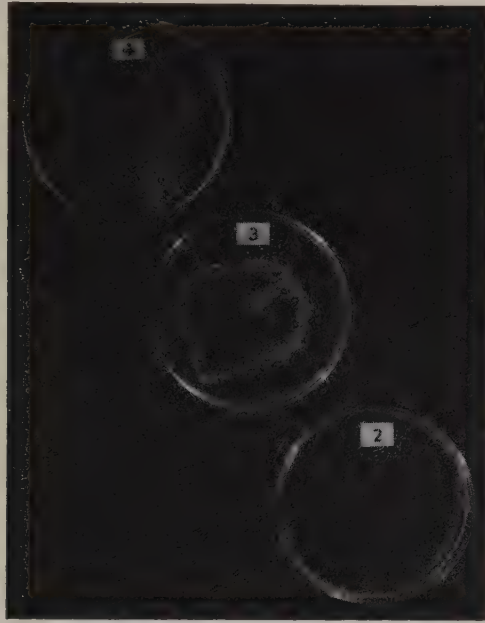


FIGURE 4. Contact plates prepared from cod fillets on the fourth day of storage at 5°C. No. 2,—interior. No. 3,—cross-section. No. 4,—surface. Surface trimethylamine nitrogen 63 mg. Interior trimethylamine nitrogen 15 mg. per 100 g.

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SUMMARY

The contact plate method for estimating the relative numbers of bacteria has shown that the surface of the fillet is heavily contaminated from the outset, while few bacteria could be demonstrated on samples from the interior, even after considerable spoilage had taken place on the surface.

Similarly, chemical tests (trimethylamine value, tyrosine value, and pH value), have shown that the spoilage changes begin at the surface and proceed there at a much greater rate than in the interior.

In the light of this surface concept of spoilage it has been suggested that determinations designed to detect incipient spoilage in fish fillets should be based on samples taken from the surface of the product rather than on composite ones as are ordinarily employed for chemical analysis.

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The Effect of Sodium Nitrite on Experimental Animals

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ABSTRACT

Incorporation of sodium nitrite in the diet of cats and white rats on the basis of an average sized man consuming 1 lb. (454 g.) of fish containing 0.2 per cent (908 mg.) of this salt daily for six days each week does not appear to affect their growth rate nor the development (weight) of their thyroid, heart, lungs, spleen, liver, kidneys or adrenals. The fecundity of white rats as judged by their ability to breed and raise normal litters is apparently not affected thereby. The lethal dose of sodium nitrite by oral route is about 1.1 to 2.0 g./kg. for healthy male rats, 0.46 to 1.2 g./kg. for healthy female rats and 0.073 g./kg. for cats (one animal). The lethal dose by subcutaneous route is about 0.19 to 0.20 g./kg. for healthy male rats and 0.057 to 0.13 g./kg. for healthy female rats.

INTRODUCTION

Recent work has shown that nitrites inhibit bacterial spoilage of fish (Tarr and Sunderland 1940) and that under certain conditions they are also able to exert a pronounced bacteriostatic and bactericidal action (Tarr 1941a and b, 1942). The present regulations in Canada (Food and Drugs Act, Ottawa, 1939) permit the incorporation of sodium nitrite in amounts not exceeding 0.02 per cent in *cured* meats (including fish, shellfish, etc.). In view of these facts and the knowledge that nitrite salts are regularly consumed by those who partake of cured meats, it was thought advisable to ascertain what effect continued oral administration of sodium nitrite in sub-lethal amounts might have on experimental animals, with special consideration of its possible cumulative toxic effect. A few experiments were also undertaken to estimate the lethal dose.

It is well known that nitrites, both organic and inorganic, act as vasodilators and anti-spasmodics, their action in small doses being to dilate the capillaries and thus to reduce the blood pressure. Both sodium nitrite and amyl nitrite have been used for years in the treatment of certain heart conditions (Dixon 1921). According to Reiss, Meyer and Müller (1928) the German Pharmacopeia lists 1 g. of sodium nitrite as the maximum permissible daily dose and the maximum single dose is stipulated as 0.04 to 0.08 g. Excessive doses of nitrites have a narcotic effect on the nervous system, produce cold shivers, dizziness, vertigo, ringing in the ears, suffocating feeling, vomiting, cyanosis, methaemoglobinaemia and possibly death through a process analogous to asphyxiation.

It has been stated that at least 0.2 g. must be consumed orally in one dose to produce the mildest symptoms in adults, slightly less than this amount being tolerated safely (Reiss *et al.* 1928).

Cases of nitrite poisoning resulting from the consumption of cured meats do not appear to be of very frequent occurrence. Thus Behre (1939) stated that, "Trotzdem Pökelfleisch sowohl das in salpeter- und zuckerhaltiger als auch in das in nitrithaltiger Pökellake hergestellte Fleisch, in ungeheurer grosser Menge genossen wird, ist bisher in Deutschland kein Fall bekanntgeworden in dem Vergiftung durch den Genuss solchen Fleisches mit Sicherheit festgestellt worden ware." He described a few rather mild and non-fatal cases of nitrite poisoning in a German family due to consumption of "pickled ribs". Several cases of fatal poisoning due to consumption of sodium nitrite in apparent mistake for ordinary table salt have been recorded (Scholes 1936, Hunziter-Kramer 1936, Padberg and Martin 1939).

EXPERIMENTAL

EFFECT OF CONTINUED ORAL DOSES

In the following two experiments growing animals were fed daily except Sunday for some months a diet incorporating weighed amounts of a flesh supplement containing 0.2 per cent of sodium nitrite (ten times the legal concentration). Control animals were fed a corresponding diet with nitrite-free supplement. The amounts of supplement fed were adjusted periodically to be proportional to the maximum weights of fish considered likely to be consumed *per diem* by a normal human being at corresponding stages of growth (table I).

TABLE I. Amount of sodium nitrite administered orally to experimental animals with changing body weight.

Man (hypothetical case)		White rats		Cats	
Weight (g.)	Wt. NaNO ₂ ingested (g.)	Weight (g.)	Wt. NaNO ₂ ingested (g.)*	Weight (g.)	Wt. NaNO ₂ ingested (g.)*
13,600	0.226	30	0.005	790	0.0226
31,800	0.454	70	0.010	1930	0.0454
49,900	0.680	110	0.015	2940	0.0680
68,000	0.908	150	0.020	**4080	0.0908

*The amount of sodium nitrite fed was not increased until animals attained the next highest weight. Thus rats which weighed between 30 and 70 g. were fed 0.005 g. of NaNO₂.

**None of the cats studied attained this weight prior to the conclusion of the experiment.

The nitrite-containing supplement for albino rats was prepared from freshly-opened canned pink salmon which, after thorough mixing with 0.2 per cent of

sodium nitrite, was stored in sealed $\frac{1}{4}$ -lb. cans held at -20°C . The nitrite content of the stored material was found not to alter appreciably during the period of the experiments. The nitrite-containing supplement for cats was prepared by thoroughly mixing the appropriate amount of a 20 or 40 per cent solution of sodium nitrite with 15 to 20 g. of finely divided flesh (not necessarily fish) immediately before feeding.

EXPERIMENT 1 (WHITE RATS)

Five brother and four sister pairs were used (three pairs from each of three litters, A, B and C). Initial weights varied from 29 to 66 g. (males) and from 26 to 60 g. (females). One of each pair was fed nitrite-containing fish, the other (control) fish without nitrite. They were segregated in six groups, each of three animals (two males and one female for each of litters A and B, and two females and one male for litter C). The feeding experiments lasted 127 to 168 days in different instances.

Except as undernoted, the animals were given free access to water and an unlimited quantity of a stock diet mixed in the following proportions: ground (whole) wheat 3000 g., ground "Purina" fox chow 1000 g., sodium chloride 50 g., dried brewer's yeast 100 g., dogfish liver oil 100 g., kelp meal 50 g., and dried "Trumilk" 750 g. In addition they received daily small amounts of one or more of the following: raw or cooked beef liver, raw carrot, apple, orange, or green vegetable.

The supplemental fish ration was controlled as follows. Every day all food and water was removed from the cages for a period varying from 2 to 5 hours. Each animal was then transferred to a separate cage, the weighed amount of fish was introduced on a metal dish, and the animal was watched until the entire ration was consumed, usually voraciously. The animal was then returned to its cage and to its regular diet.

The animals matured, mated, and the females raised one or more litters. They were at first weighed bi-weekly, later once a week, to the nearest gram. Females were isolated shortly before littering and were returned to the males subsequent to weaning of the young. Some first litters died through lack of adequate development of the mothers' mammary glands (figure 1). One second litter (of a control) was lost through this cause. The young were almost all healthy and of average weight, there being no perceptible difference between nitrite-fed animals and controls in this respect.

The sole symptom attributable to nitrite feeding was shown by three litter C rats which, during the first 10 days of the experiment, exhibited slight transient respiratory distress, lasting 30 minutes or less.

Growth curves (shown for each pair in figure 1) indicated no effect of nitrite on growth-rate. Fecundity was about the same, 53 young from nitrite-fed mothers, as against 58 from controls. The offspring from the nitrite-fed animals appeared more resistant, only 30 per cent dying before weaning, as against 52 per cent of the young of the controls, but in view of the smallness of the number of animals this difference is probably not significant.

At the end of the feeding experiment the rats were killed by chloroform, and

frozen at -20°C . Prior to dissection they were only partially thawed, so that the liver and heart remained filled with blood. Thyroid, heart, lungs, liver, spleen, kidneys and adrenals were dissected out and weighed (Cameron 1925).

The extremes and mean organ weights for each group of animals are shown in table II, and exhibit no essential difference between experimental animals and controls. The figures are in general of the same order as those reported by Cameron and Sedziak (1921) and by Cameron (1925) though, owing to the blood content, those for hearts are uniformly higher.

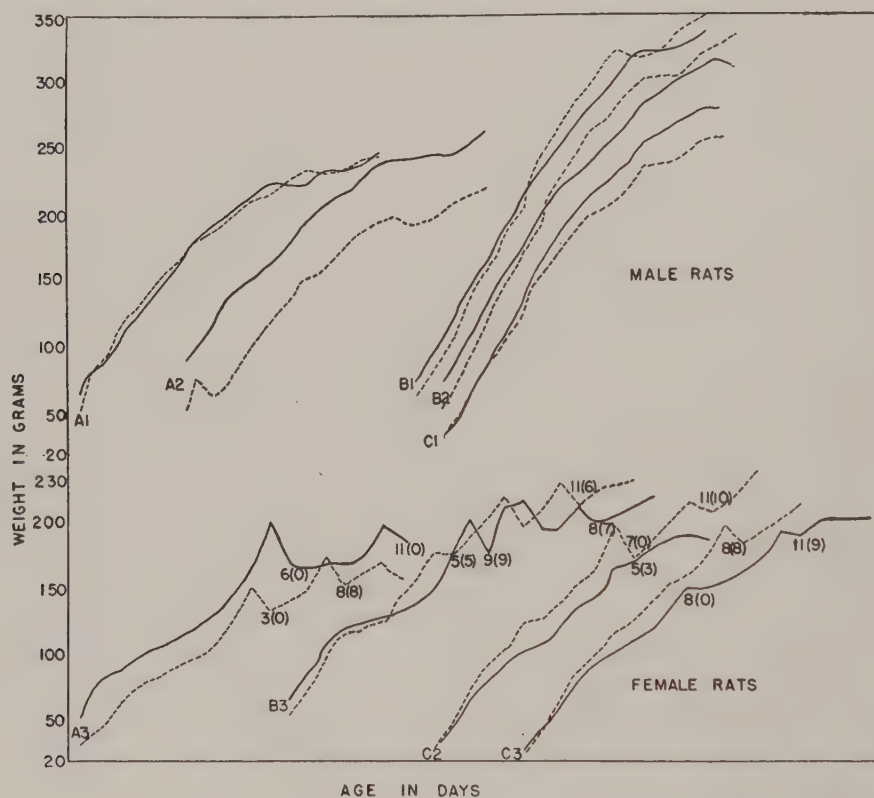


FIGURE 1. Growth curves of white rats fed diets with (broken lines) and without (continuous lines) added sodium nitrite (experiment 1). The figures along the female growth curves represent the number of young in litters, the figures in parentheses showing the number of young successfully reared and weaned in the litter.

Hence the regular feeding of sodium nitrite in the described amounts over a long period does not appear to affect the health, rate of growth, size of body organs, fecundity, and health of offspring of the white rats. It may be noted that a typical animal in this experiment received in 121 days 0.167 g. of sodium nitrite, an amount almost 50 per cent greater than the maximum non-lethal single oral dose indicated for the rat in experiment 3.

TABLE II. Comparison of organ weights of nitrite-fed and control rats.

Data obtained	4 females (nitrite-fed)	4 females (controls)	5 males (nitrite-fed)	5 males (controls)
Total body weight (g.)				
Initial.....	27-56	26-60	29-66	31-63
Final				
Extremes.....	186-229	183-217	218-350	243-335
Mean.....	210	204	280	285
Organ weight (%):				
Kidneys				
Extremes.....	1.00-1.09	0.98-1.11	0.81-0.95	0.82-1.05
Mean.....	1.05	1.07	0.90	0.94
Liver				
Extremes.....	7.3-8.8	6.5-7.3	4.0-5.1	4.1-5.0
Mean.....	7.7	6.6	4.7	4.6
Heart				
Extremes.....	0.84-1.06	0.76-1.05	0.63-1.01	0.64-0.79
Mean.....	0.97	0.87	0.70	0.73
Spleen				
Extremes.....	0.18-0.23	0.16-0.38	0.16-0.27	0.18-0.26
Mean.....	0.21	0.28	0.22	0.22
Thyroid				
Extremes.....	0.0058-0.0081	0.0054-0.0078	0.0039-0.0064	0.0052-0.0069
Mean.....	0.0072	0.0069	0.0053	0.0059
Lungs				
Extremes.....	0.79-1.20	0.77-0.93	0.75-1.00	0.74-1.04
Mean.....	0.94	0.82	0.81	0.83
Adrenals				
Extremes.....	0.025-0.032	0.027-0.028	0.0086-0.013	0.0091-0.013
Mean.....	0.028	0.028	0.011	0.011

EXPERIMENT 2 (CATS)

Five kittens from three different litters were used, two being retained as controls and the others being fed sodium nitrite in their diet (table III). They were kept in a large wire enclosure in a room at uncontrolled temperature (3 to 15°C.). Their diet consisted of a liberal quantity of milk ("Trumilk") and one of the following foods which were rarely fed for more than two days successively: cooked beef liver or heart, cooked fish (several varieties), "Pet" canned animal food kindly donated by the B.C. Packers Ltd., and a small quantity of mixed fish liver oils as source of vitamins A and D. The animals were normally fed in the morning and in the afternoon, no food being given for from 12 to 16 hours prior to isolating and feeding individual animals their nitrite-containing supplement.

Six days after the commencement of the experiment the animals were treated for worms, a single capsule containing 0.2 ml. of tetrachloroethylene (Parke Davis) being administered orally to each animal. This treatment, when coupled with the fact that during the first few days of the experiment the rather large

food requirement of the animals was not fully met, undoubtedly accounts for the fact that most of them either failed to gain weight or lost a little during the first week. No unusual external symptoms were observed in cats which received the nitrite-containing supplement.

The cats were normally weighed each week, and after 15 weeks were chloroformed, frozen, partially defrosted and their organs dissected out and weighed as in experiment 1. The organ weights of a 7- to 8-months-old female cat from a different litter which was killed by a single large dose of sodium nitrite (see experiment 4) have been included in this work.

The results (figure 2 and table III), although based on a very small number of experimental animals, strongly indicated that the regular feeding of sodium nitrite in the described amounts resulted in no important differences in either the

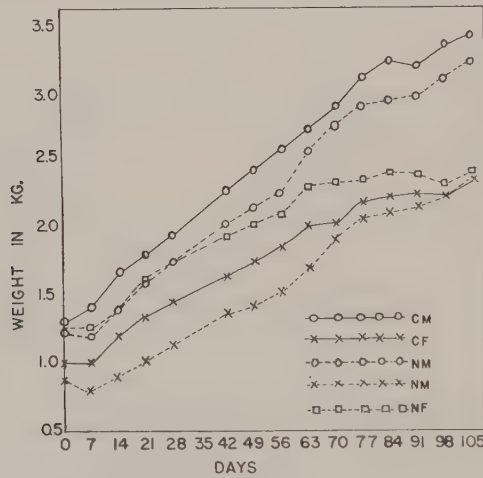


FIGURE 2. Growth curves of cats fed diets with (broken lines) and without (continuous lines) added sodium nitrite (experiment 2).

TABLE III. Comparison of organ weights of nitrite-fed and control cats.

Initial wt. (g.)	Final wt. (g.)	Body weight (%)						
		Kidneys	Liver	Heart	Spleen	Thyroid	Lungs	Adrenals
1300 CM	3400	1.04	4.5	0.87	0.23	0.0061	1.3	0.012
1010 CF	2340	0.88	4.5	0.84	0.18	0.0068	1.2	0.012
1230 NM	3200	0.79	4.6	0.75	0.14	0.0055	1.0	0.010
855 NM	2370	1.2	5.1	0.93	0.20	0.0089	1.5	0.015
1270 NF	2410	0.89	5.2	0.86	0.15	0.0071	0.85	0.009
.... F*	2550	1.1	4.4	0.80	0.20	0.0067	0.91	0.016

*This female was killed by a lethal dose of NaNO_2 and had not received the same treatment as the other animals.

C = control, N = nitrite-fed, M = male, F = female. Second, third and fourth animals were from the same litter.

growth rate or organ weights of the nitrite-fed cats as compared with the controls. The third cat in table III ingested approximately 4.1 g. of sodium nitrite during the experiment without apparent ill effect.

LETHAL DOSE

Rappeport-Lewey (1936) found that the minimum lethal dose of sodium nitrite for rabbits was 0.0876 grams per kilogram of body weight, the definite lethal dose being about 0.12 g. per kg. Sheep are sometimes poisoned by the nitrite which arises when the nitrate of Australian mint weed is reduced enzymically (Williams and Hines 1939). Oral ingestion of 0.08 g. of sodium nitrite per kilogram produces anoxaemia in sheep, and this is rapidly corrected by administration of methylene blue solution (Scott 1941). The lethal dose of sodium nitrite for man is probably not known, but in susceptible individuals 0.0043 g. per kg. of body weight may produce marked symptoms of poisoning (Reiss *et al.* 1928). Reference has already been made to fatal cases of nitrite poisoning in adults occurring as a result of seasoning food with sodium nitrite in mistake for salt.

EXPERIMENT 3 (WHITE RATS)

Oral administration. Preliminary tests indicated that the rather large amount of sodium nitrite required to kill a rat could not readily be incorporated in its food, so solutions (10, 20 or 40 per cent by volume) were injected well down the oesophagus of the animals, using a small hypodermic equipped with a glass cannula. With care it was found that little of the liquid thus introduced was lost, though with small rats it was necessary to introduce amounts in excess of 0.1 ml. subdivided into successive doses at brief intervals. The treated animals were kept under observation and those which survived were usually retained for further tests.

The results (table IV) showed that for apparently healthy vigorous male rats the minimum lethal dose of sodium nitrite by oral route was 1.1 grams per kilogram of body weight, the maximum surviving dose being 2 g. per kg. It can therefore be assumed that the lethal dose will normally lie between these limits. For female rats the corresponding limits were 0.46 and 1.2 g. per kg. That the toxic effect of sodium nitrite in this experiment was not due to the introduction of rather large quantities of hypertonic salt solution is shown by the fact that oral introduction of sodium *nitrate* in amounts as great as 4 g. per kg. was in no case fatal. The results showed that old feeble rats were much more susceptible to sodium nitrite than were young vigorous rats.

Subcutaneous injection. A small patch of hair over the biceps femoris muscle of the hind leg was removed and the skin washed with tincture of phenyl mercuric nitrate (Birkhaugh 1933). Solutions of sodium nitrite (1, 10 or 20 per cent by volume and sterilized by Seitz filter) were injected subcutaneously into a number of rats of different sex and age, using a small hypodermic and a no. 25 needle (table V). Male rats recovered after injection of from 0.03 to 0.19 g. of sodium nitrite per kilogram, but died when from 0.20 to 0.48 g. per kg. were administered; the lethal dose therefore appeared to lie between 0.19 and 0.20 g. per kg. Females appeared to be more sensitive than males. They recovered when from

TABLE IV. Lethal dose of sodium nitrite for white rats by oral route.

Weight (g.) and sex	Solution administered		Amount of NaNO ₂ or NaNO ₃ administered (mg.)	Amount of NaNO ₂ or NaNO ₃ introduced (g. per kg. of body wt.)	Result
	Amount (ml.)	Concen- tration (%)			
230 M	NaNO ₂ 0.20	NaNO ₂ 40	80	0.35	Survived
*142 M	0.20	"	60	0.42	"
86 F	0.10	"	40	0.46	Died in 3 hr. 37 min.
40 F	0.05	"	20	0.50	Survived
216 M	0.30	"	120	0.55	"
71 M	0.20	20	40	0.56	"
34 F	0.05	40	20	0.58	"
207 M	0.30	"	120	0.58	"
*133 M	0.20	"	80	0.60	"
+ 32 F	0.10	20	20	0.62	"
** 65 F	0.20	"	40	0.62	"
44 M	0.15	"	30	0.68	"
140 F	0.30	40	120	0.86	Died in 29 min.
42 F	0.20	20	40	0.95	Survived
+ 36 M	0.20	"	40	1.1	"
76 M	0.20	40	80	1.1	Died in 28 min.
34 F	0.10	"	40	1.2	Died in 40 min.
34 M	0.10	"	80	1.2	Survived
** 66 F	0.40	20	80	1.2	"
* 98 M	0.30	40	120	1.2	Died in 1 hr. 4 min.
30 M	0.10	"	40	1.3	Survived
+ 30 M	0.10	"	40	1.3	Died in 10 min.
47 M	0.30	20	60	1.3	Died in 2 hr. 20 min.
81 M	0.30	40	120	1.5	Died in 22 min.
35 F	0.15	"	60	1.7	Died in 12 min.
45 F	0.20	"	80	1.8	Died in 13 min.
+ 40 M	0.20	"	80	2.0	Survived
40 F	0.40	20	80	2.0	Died in 26 min.
+ 38 M	0.20	40	80	2.1	Died in 3 hr.
40 M	0.50	20	100	2.5	Died in 2 to 3 min.
29 F	0.20	40	80	2.8	Died in 11 min.
** 64 M	0.50	"	200	3.1	Died in 8 min.
22 M	0.30	"	120	4.0	Died in 8 min.
** 38 M	0.40	"	160	4.2	Died in 10 min.
±222 F	0.20	20	40	0.18	Died in 3 hr. 30 min.
±248 M	0.40	"	80	0.32	Died in 1 hr. 30 min.
±229 F	0.30	40	120	0.52	Died in 1 hr. 45 min.
	NaNO ₃	NaNO ₃			
92 F	0.30	40	120	1.3	Survived
32 F	0.20	"	80	2.5	"
40 F	0.30	"	120	3.0	"
44 F	0.40	"	160	3.6	"
29 F	0.30	"	120	4.0	"

*Animals which had recovered from a sub-lethal dose of sodium nitrite administered at least 7 days before, and which were apparently healthy.

**Animals which had recovered from a sub-lethal dose of sodium nitrite administered at least 7 days before, were apparently healthy and had been starved from 16 hours prior to administering the above nitrite solution.

+ Starved for 5 hours prior to administration of nitrite solution.

± Old feeble rats.

TABLE V. Lethal dose of sodium nitrite for white rats by subcutaneous route.

Weight of rat (g.) and sex	Solution injected		Amount of NaNO ₂ or NaNO ₃ injected (mg.)	Amount of NaNO ₂ or NaNO ₃ introduced (g. per kg. body wt.)	Result
	Amount (ml.)	Concentration (%)			
	NaNO ₂	NaNO ₂			
73 F	0.10	1	1	0.014	Survived
26 M	0.08	1	0.08	0.034	"
138 F	0.025	20	5	0.036	"
112 F	0.05	10	5	0.045	"
*106 F	0.03	20	6	0.057	"
70 M	0.05	10	5	0.072	"
26 M	0.02	"	2	0.077	"
105 M	0.1	"	10	0.095	"
* 94 M	0.05	20	10	0.11	"
33 M	0.02	"	4	0.12	"
61 F	0.04	"	8	0.13	Died in 1 hr. 25 min.
29 M	0.04	10	4	0.14	Survived
62 F	0.05	20	10	0.16	Died in 43 min.
27 M	0.05	10	5	0.19	Survived
*101 M	0.10	20	20	0.20	Died in 34 min.
33 M	0.04	"	8	0.24	Died in 1 hr. 12 min.
83 M	0.2	10	20	0.24	Died in 17 min.
104 F	0.15	20	30	0.29	Died in 24 min.
33 M	0.08	"	16	0.48	Died in 27 min.
	NaNO ₃	NaNO ₃			
123 M	0.20	20	40	0.33	Survived
30 M	0.08	"	16	0.53	"
61 M	0.25	"	50	0.82	"

*Animals which had recovered from a sub-lethal dose of sodium nitrite administered at least 7 days before and which were apparently healthy.

0.014 to 0.057 g. per kg. were injected, and died after administration of from 0.13 to 0.29 g. per kg., the lethal dose therefore lying between 0.057 and 0.13 g. per kg. Thus a much larger dose of nitrite was required to kill a rat when introduced orally than when injected subcutaneously. Subcutaneous injection of from 0.33 to 0.82 g. of sodium *nitrate* per kilogram did not cause any important reaction in rats.

EXPERIMENT 4 (CAT)

An apparently normal healthy female cat maintained on the usual mixed diet was fed at intervals increasing amounts of sodium nitrite incorporated in approximately 30 g. of finely minced cooked liver or fish, after starving for some hours as in experiment 2. The results were as follows:

Day	Weight (kg.)	Mg. of NaNO ₂ fed	Reaction
0	3.34	80	None observed
2		160	" "
4		180	Vomited 80 minutes after feeding; suffered severe anoxaemia and was unable to stand. Vomited a second time about 20 minutes later. Four hours after the nitrite was administered the animal had apparently recovered completely.
18	3.28	240	Vomited about 1½ hours after feeding, suffered severe anoxaemia and cyanosis and died about 2 hours after ingesting the nitrite.

This experiment indicates that about 0.073 g. of sodium nitrite per kilogram will kill a cat when introduced orally with its food, assuming that previous administration of the salt in sub-lethal amounts did not in any way influence the final reaction of the animal.

Another female cat weighing 2.55 kg. and about 7 to 8 months old was fed 400 mg. of sodium nitrite in approximately 40 to 50 g. of chopped liver. The animal vomited about 10 minutes after feeding, suffered from severe anoxaemia and cyanosis, and died about 1 hour after the nitrite was ingested.

DISCUSSION

These experiments indicate that the growth rate and weight of certain internal organs of cats and white rats, and the fertility of the latter, are not appreciably affected by continued oral administration of sub-lethal quantities of sodium nitrite.

Brooks (1937) showed that *in vitro* in the absence of oxygen and the presence of a reducing agent, haemoglobin was converted quantitatively into nitroso-haemoglobin, the reaction proceeding much more rapidly at pH 5.15 to 6.63 than at pH 7.16 to 7.75. In the absence of a reducing agent one molecule of sodium nitrite combined with two equivalents of reduced haemoglobin to give one equivalent each of nitrosohaemoglobin and methaemoglobin. According to Brooks it is probable that in the presence of oxygen oxyhaemoglobin reacts with sodium nitrite to form nitrosohaemoglobin, methaemoglobin and possibly other compounds. The usual product of the *in vivo* interaction of sodium nitrite and haemoglobin is apparently methaemoglobin (Rappeport-Lewey 1936, Padberg and Martin 1939, Cox and Wendel 1942), though Hunziter-Kramer (1936) stated that in some fatal human cases of nitrite poisoning, the blood contained no methaemoglobin but was bright red in colour and may therefore have contained some nitrosohaemoglobin.

Recent experiments by Cox and Wendel (1942) support the contention that continued oral administration of nitrite is probably not harmful. They found that prolonged methaemoglobinaemia in a dog which received intravenously 30 mg. of sodium nitrite per kilogram of body weight at intervals of 5 to 6 hours (7 injections over a 36-hour period) had no apparent effect on the ability of the

natural reducing systems of the body to reduce the methaemoglobin so formed. In this experiment 52 to 65 per cent of the total haemoglobin was maintained in the form of methaemoglobin over the 36-hour period, and they calculated that the total blood pigment must have undergone reduction and oxidation at least four times. The average rate at which methaemoglobin was reduced *in vivo* to functionally active haemoglobin in dogs was 11.3 ± 2.0 per cent of the total blood pigment per hour. The injection of methylene blue accelerated the reduction. Experiments with rabbits and rats have shown that the methaemoglobin formed *in vivo* slowly disappears (Gelinsky 1940).

It appears that *in vivo* nitrite itself is probably partly oxidized to nitrate or further broken down, though some may be excreted unchanged (Sollmann 1922, Dixon 1921).

Hale (1910) believed that protein digestion was impaired in the presence of small amounts of nitrite, and more recently Sleeth and van Liere (1941) have stated that nitrite delays the emptying time of the human stomach. Further experimental data are needed to confirm or disprove these statements.

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The Action of Nitrites on Bacteria: Further Experiments

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ABSTRACT

Growth of the obligate anaerobes *Clostridium botulinum* and *C. sporogenes* is inhibited by 0.02 per cent sodium nitrite in fish digest broth at acid pH, inhibition not being accentuated by brief exposure of the cultures to air during the vigorous growth phase. Sodium nitrite inhibits growth of facultative anaerobes in nutrient broth under both strictly and semi-anaerobic conditions. Growth of facultative anaerobes is inhibited by sodium nitrite over a much wider pH range in nutrient broth than in fish digest broth or in a simple synthetic ammonium lactate medium. Sodium nitrite is capable of acting in either bactericidal or bacteriostatic capacity under different conditions, and inhibits growth of the human pathogens *Eberthella typhosa* and *Staphylococcus aureus*. The experiments described throw no light on the mechanism by which sodium nitrite, or its decomposition products, inhibit bacterial growth.

In previous publications (Tarr and Sunderland 1940, Tarr 1941) it was shown that sodium nitrite inhibited growth of bacteria in culture media and in fish muscle only when these substrates possessed a somewhat acid reaction. The mechanism by which nitrite inhibited growth was not ascertained, but it was demonstrated that its action depended little, if at all, on its inhibitory effect on aerobic respiratory catalysts.

EXPERIMENTAL

CULTURES

Certain of the organisms employed were those used in previous work (Tarr 1941), culture 4a being the strain of *Pseudomonas* formerly referred to as culture 4. The following organisms, obtained from the American Collection of Type Cultures, were also used: *Staphylococcus aureus* (no. 152), *Eberthella typhosa* (no. 167), *Clostridium botulinum* (no. 687) and *Clostridium sporogenes* (no. 459).

MEDIA

FISH DIGEST BROTH

Defrosted halibut muscle was digested with Bacto trypsin following the procedure employed in preparing beef digest broth (Hartley 1922). The finished medium (pH 5.9) was stored under toluene. Prior to use portions of this medium

were adjusted to desired pH values by addition of 1.0 normal HCl or NaOH. They were then heated in flowing steam, cooled, and filtered through a Seitz filter in order to remove bacterial cells and any insoluble precipitated protein material which might interfere with direct bacterial counts (*vide infra*). Immediately prior to use, 2-ml. portions of the filtered medium were sterilized in 12 by 150-mm. culture tubes. This medium, being naturally well buffered, required no added buffer solution.

BUFFERED NUTRIENT BROTH

Eight grams of Bacto dehydrated nutrient broth were dissolved in 1 litre of distilled water and adjusted to pH 5.2 by the addition of 3.5 ml. of 1.0 normal HCl, 1.5-ml. portions being dispensed into 12 by 150-mm. culture tubes. Prior to use, 0.5 ml. of sterile 0.2 molar $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer (different pH values) was added to each tube of broth.

SYNTHETIC MEDIUM

This had the following composition: Lactic acid (Baker's A.R.) 5 ml., NaCl 1.0 g., trace of MgSO_4 and FeCl_2 , strong (28 per cent) NH_4OH to adjust the solution to pH 5.0; 1.5-ml. portions were dispensed into 12 by 150-mm. culture tubes, sterilized, and adjusted to various pH values by addition of phosphate buffer as in the case of the nutrient broth medium.

For all media employed, the pH as reported in the various tables is that of the medium as diluted by addition of nitrite solution (or water) and the inoculum. It was found that with well-buffered media of this type the addition of 0.02 per cent of NaNO_2 did not occasion a significant change in pH.

TECHNIQUE OF INOCULATION AND INCUBATION

The facultative anaerobes were grown for 16 to 24 hours in a medium (pH 7.0) of the same type as that into which they were to be inoculated. The obligate anaerobes were grown for 24 hours in fish digest broth (pH 7.0) in cases where an inoculum consisting largely of vegetative cells was required, and for 7 days in the same medium in order to obtain cultures consisting mainly of endospores. Saline suspensions of the organisms for inoculation purposes were prepared by suitably diluting such cultures with sterile 0.01 per cent NaCl solution. Suspensions containing mainly endospores of the obligate anaerobes were heated at 80°C. for 10 minutes in order to destroy any vegetative cells which might be present. In the various experiments to be described individual tubes of medium were each inoculated with 0.5 ml. of a saline suspension of the organism being studied. Preparation and addition of NaNO_2 solutions to the various media, pH measurements, direct and viable bacterial counts and examination of inoculated cultures were carried out as described in previous publications (Tarr 1941, Tarr and Bailey 1939). Cultures of *C. botulinum*, *C. sporogenes*, *S. aureus* and *E. typhosa* were incubated at 37°C., the remainder being incubated at 25°C. Strictly anaerobic conditions were maintained by using McIntosh and Fildes' jars, employing the technique of removing some of the air by partial evacuation prior to letting in hydrogen and carrying out the combustion.

INHIBITION IN FISH DIGEST BROTH

OBLIGATE ANAEROBES

Experiment 1. Each tube of one set of fish digest broth medium was inoculated with approximately 17.7×10^6 *C. botulinum* organisms (largely vegetative cells or sporulating vegetative cells), the rate of growth being determined at intervals. The results of this experiment (table I) show that 0.02 per cent NaNO_2 markedly inhibited growth of this organism at pH values below 7.0, multiplication being entirely prevented at pH 4.6 to 5.6 even after 14 days.

Experiment 2. Two sets of fish digest broth were inoculated with *C. botulinum*, each tube receiving approximately 2.5×10^6 spores. One set was incubated anaerobically for 14 days, the other being similarly treated except that after the first day the medium was in each case exposed to air and shaken well in order to make bacterial counts, thus permitting access of air. The results (table II), show that 0.02 per cent NaNO_2 inhibited growth in both cases. It will be seen that, in the cultures exposed to air, growth did not occur below pH 6.1, while in the foregoing experiment under similar conditions a larger inoculum consisting mainly of vegetative cells caused growth even at pH 4.6. When the two sets are compared it will be seen that growth occurred at a much lower pH in both control and nitrite-treated cultures which were not exposed to air.

Experiment 3. Each tube of a set of fish digest broth was inoculated with approximately 19.6×10^6 *C. sporogenes* organisms (largely vegetative cells or sporulating vegetative cells), the rate of growth being determined at intervals

TABLE I. Growth of *C. botulinum* in fish digest broth of different pH with and without 0.02 per cent NaNO_2 . (Inoculum 17.7×10^6 organisms, mainly vegetative cells; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after the following incubation periods							
	1 day		2 days		7 days		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
3.60	0	0	0	0	0	0	0	0
4.10	0	0	0	0	0	0	0	0
4.60	0	0	0	0	+	0	+	0
5.10	0	0	0	0	+	0	+	0
5.60	0	0	0	0	+	0	+	0
6.10	0	0	257	0	+	+	+	+
6.60	80	4	306	0	+	+	+	+
7.05	360	5.5	*155	180	+	+	+	+
7.50	296	330	*117	*119	+	+	+	+
7.80	224	219	* 36	* 60	+	+	+	+

*Decrease due to autolysis of vegetable cells; mainly spores remaining.

0 = No visible growth or increase in original inoculum.

+

TABLE II. Growth of *C. botulinum* in fish digest broth with and without 0.02 per cent NaNO_2 (Inoculum 2.5×10^8 spores; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Medium shaken in presence of air in making direct counts on first day of experiment				Completely anaerobic conditions throughout experiment	
	Growth observed after the following incubation periods				Growth observed after incubating for 14 days	
	1 day		14 days		Control	0.02% NaNO_2
	Control	0.02% NaNO_2	Control	0.02% NaNO_2		
3.60	0	0	0	0	0	0
4.10	0	0	0	0	+	0
4.60	0	0	0	0	0	+
5.10	0	0	0	0	+	0
5.60	0	0	0	0	+	+
6.10	3	0	+	0	+	+
6.60	125	0	+	0	+	+
7.05	225	0	+	+	+	+
7.50	230	226	+	+	+	+
7.80	219	202	+	+	+	+

and the organisms thus being exposed to air. The results (table III) show that nitrite inhibited growth of this organism, the general effect being similar to that obtained in experiment 1 with *C. botulinum*.

Experiment 4. Two sets of fish digest broth were inoculated with approximately 5.2×10^6 spores of *C. sporogenes* and treated as in experiment 2. The results (table IV) were very similar to those obtained in the case of *C. botulinum*. They show that large inocula consisting chiefly of vegetative cells (compare with table III) caused growth at lower pH values than did small inocula of spores, and that growth occurred at much lower pH values in cultures not exposed to air than in those exposed thereto. Nitrite inhibited growth over about the same range of pH in cultures exposed, or not exposed, to air.

It will be noticed that in certain of the experiments with anaerobes somewhat irregular results were obtained in the case of cultures not exposed to air during growth (see tables II and IV). Thus growth in one instance occurred at pH 4.6 but not at either pH 4.1 or at pH values between 5.1 and 6.1. The reason for this is not clear, but it is not altogether unexpected, for it is known that obligate anaerobes are rather fastidious with respect to their nutrient requirements, even small amounts of CO_2 being required for growth (Knight 1941). Minor differences in CO_2 content of the medium might thus account for the observed irregularity.

FACULTATIVE ANAEROBES

Experiment 5. One set of fish digest broth was inoculated with each of three

TABLE III. Growth of *C. sporogenes* in fish digest broth of different pH with and without 0.02 per cent NaNO_2 (Inoculum 19.6×10^6 organisms, mainly vegetative cells; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after the following incubation periods							
	1 day		2 days		7 days		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
3.60	0	0	0	0	0	0	0	0
4.10	0	0	0	0	0	0	0	0
4.60	0	0	0	0	0	0	0	0
5.10	0	0	0	0	0	0	+	0
5.60	0	0	189	0	+	0	+	0
6.10	0	0	209	0	+	0	+	0
6.60	0	0	288	0	+	0	+	0
7.05	0	0	307	0	+	0	+	+
7.50	20	7	198	152	+	+	+	+
7.80	40	0	173	203	+	+	+	+

TABLE IV. Growth of *C. sporogenes* in fish digest broth of different pH with and without 0.02 per cent NaNO_2 (Inoculum 5.2×10^6 spores; figures for growth are in millions per ml. by direct count)

Initial pH of medium	Medium shaken in presence of air in making direct counts on first day of experiment				Completely anaerobic conditions throughout experiment	
	Growth observed after the following incubation periods				Growth observed after incubating for 14 days	
	1 day		14 days		Control	0.02% NaNO_2
	Control	0.02% NaNO_2	Control	0.02% NaNO_2		
3.60	0	0	0	0	0	0
4.10	0	0	0	0	+	0
4.60	0	0	0	0	+	+
5.10	0	0	0	0	+	0
5.60	5	0	0	0	+	0
6.10	25	0	+	0	+	0
6.60	77	0	+	0	+	+
7.05	227	0	+	0	+	+
7.50	239	3	+	+	+	+
7.80	233	136	+	+	+	+

different organisms as follows: (1) *S. aureus*, 1.6×10^6 organisms per tube of medium. (2) Culture 6 (*Flavobacterium* sp.), 4.8×10^6 organisms per tube of medium. (3) Culture 9 (*Achromobacter* sp.), 1.6×10^6 organisms per tube of

medium. The tubes were incubated as usual, bacterial counts being made at intervals. The results (tables V, VI, and VII) show that NaNO_2 retarded growth of all three organisms in medium of acid pH values. However, the retarding or inhibiting effect of the nitrite was much less marked, and did not extend over nearly such a wide range of pH as was observed when the same or different organisms were cultivated in ordinary nutrient broth (compare tables VIII to XIII).

TABLE V. Growth of *S. aureus* in fish digest broth of different pH with and without 0.02 per cent NaNO_2 (Inoculum 1.6×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after					
	6 hours		1 day		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
3.60	0	0	0	0	0	0
4.10	0	0	0	0	0	0
4.60	0	0	10.5	0	+	0
5.10	0	0	66	0	+	0
5.60	3.8	0	225	67	+	+
6.10	39	20	374	340	+	+
6.60	51	45	400	413	+	+
7.05	54	49	394	402	+	+
7.50	49	51	302	292	+	+
7.80	56	54	253	259	+	+

TABLE VI. Growth of culture 6 (*Flavobacterium* sp.) in fish digest broth of different pH with and without 0.02 per cent NaNO_2 (Inoculum 4.8×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after					
	1 day		2 days		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
3.60	0	0	0	0	0	0
4.10	0	0	0	0	0	0
4.60	0	0	0	0	0	0
5.10	0	0	0	0	0	0
5.60	9	0	40	0	+	0
6.10	34	0	75	0	+	0
6.60	43	32	72	68	+	+
7.05	42	31	80	83	+	+
7.50	44	28	83	85	+	+
7.80	31	32	82	81	+	+

TABLE VII. Growth of culture 9 (*Achromobacter* sp.) in fish digest broth of different pH with and without 0.02 per cent NaNO_2 (Inoculum 1.6×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after							
	6 hours		1 day		2 days		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
3.60	0	0	0	0	0	0	0	0
4.10	0	0	0	0	0	0	0	0
4.60	0	0	0	0	0	0	0	0
5.10	0	0	0	0	0	0	0	0
5.60	0	0	0	0	316	0	+	0
6.10	1.8	0	208	0	604	150	+	+
6.60	9.8	5.2	337	332	+	+	+	+
7.05	7.9	8.5	360	358	+	+	+	+
7.50	5.6	8.4	385	379	+	+	+	+
7.80	7.7	8.1	350	358	+	+	+	+

INHIBITION IN NUTRIENT BROTH AND IN SYNTHETIC MEDIUM

Only certain of the organisms available were able to grow in the simple synthetic medium employed, the few strains of *Micrococcus* studied showing no evidence of growth. The cultures used were transferred twice in the synthetic medium prior to diluting them with saline solution and using them to inoculate the media.

Experiment 6. One set each of synthetic medium and of nutrient broth was inoculated separately with different organisms as follows: *Culture 9* (*Achromobacter* sp.), 3.6×10^6 organisms per tube of medium; *Culture 17* (*Achromobacter* sp.), 7.8×10^6 organisms per tube of medium; *Culture 4a* (*Pseudomonas* sp.), 2.7×10^6 organisms per tube of medium.

The cultures were incubated and determinations of the rate of growth were made as usual. The results (tables VIII, IX and X) show that growth of all three organisms was inhibited by NaNO_2 both in nutrient broth and in the synthetic medium. However, in every case growth occurred at a much more acid reaction, and nitrite inhibited over a much wider pH range, in nutrient broth than in the synthetic medium. Somewhat unexpected results were obtained with culture 4a when cultivated in the synthetic medium, for it was found that only at pH 5.80 did nitrite inhibit growth permanently. At pH values between 6.18 and 6.85 nitrite actually accelerated growth. The reason for this finding is not clear, but it is suggested that it may have been due to the fact that the nitrite acted as an additional hydrogen acceptor at certain pH values, thereby favouring growth. It is of interest in this connection to note that Meiklejohn (1940) showed that certain species of *Pseudomonas* carried out denitrification under aerobic as well as under anaerobic conditions, and that they reduced nitrate to nitrite and further to gaseous nitrogen.

TABLE VIII. Growth of culture 9 (*Achromobacter* sp.) in nutrient broth and in synthetic medium at different pH and with and without 0.02 per cent NaNO_2 . (Inoculum 3.6×10^6 organisms; figures for growth are in millions per ml. by direct count).

Nutrient broth							Synthetic medium						
Initial pH of medium	Growth after						Initial pH of medium	Growth after					
	18 hours		2 days		14 days			18 hours		2 days		14 days	
	Control	0.02% NaNO ₂	Control	0.02% NaNO ₂	Control	0.02% NaNO ₂		Control	0.02% NaNO ₂	Control	0.02% NaNO ₂	Control	0.02% NaNO ₂
5.12	0	0	41	0	+	0	4.44	0	0	0	0	0	0
5.42	4	0	190	0	+	0	4.48	0	0	0	0	0	0
5.58	39	0	388	0	+	0	4.54	0	0	0	0	0	0
5.88	126	0	368	0	+	0	4.60	0	0	0	0	0	0
6.10	46	4	372	35	+	+	4.85	0	0	0	0	0	0
6.30	152	60	390	380	+	+	5.20	2	0	0	0	+	0
6.50	150	106	384	360	+	+	5.80	89	0	188	0	+	0
6.72	154	145	369	378	+	+	6.18	89	3.3	251	44	+	+
6.88	144	150	377	160	+	+	6.42	98	39	305	194	+	+
7.09	140	142	359	388	+	+	6.70	95	85	300	304	+	+
7.30	137	139	398	402	+	+	6.85	91	84	296	292	+	+

TABLE IX. Growth of culture 4a (*Pseudomonas* sp.) in nutrient broth and in synthetic medium at different pH and with and without 0.02 per cent NaNO_2 . (Inoculum 2.7×10^6 organisms; figures for growth are in millions per ml. by direct count).

Nutrient broth							Synthetic medium						
Growth after							Growth after						
Initial pH of medium	18 hours		2 days		14 days		Initial pH of medium	18 hours		2 days		14 days	
	Control	0.02% NaNO ₂	Control	0.02% NaNO ₂	Control	0.02% NaNO ₂		Control	0.02% NaNO ₂	Control	0.02% NaNO ₂	Control	0.02% NaNO ₂
5.12	261	0	780	0	+	0	4.44	0	0	0	0	0	0
5.42	285	0	762	0	+	0	4.48	0	0	0	0	0	0
5.58	295	0	730	0	+	+	4.54	0	0	0	0	0	0
5.88	262	17	770	246	+	+	4.60	0	0	0	0	0	0
6.10	277	85	830	530	+	+	4.85	0	0	0	0	0	0
6.30	267	223	798	690	+	+	5.20	0	0	0	0	0	0
6.50	299	306	805	746	+	+	5.80	0	0	37	0	+	0
6.72	315	285	840	808	+	+	6.18	0	8	160	37	+	+
6.88	287	265	806	842	+	+	6.42	2	29	67	98	+	+
7.09	302	266	820	765	+	+	6.70	2	37	82	265	+	+
7.30	318	292	790	770	+	+	6.85	0	54	46	354	+	+

TABLE X. Growth of culture 17 (*Achromobacter* sp.) in nutrient broth and in synthetic medium at different pH and with and without 0.02 per cent NaNO_2 . (Inoculum 7.8×10^6 organisms; figures for growth are in millions per ml. by direct count).

Nutrient broth					Synthetic medium				
Initial pH of medium	Growth after				Initial pH of medium	Growth after			
	1 day		14 days			1 day		14 days	
	Control	0.02% NaNO ₂	Control	0.02% NaNO ₂		Control	0.02% NaNO ₂	Control	0.02% NaNO ₂
5.12	57	0	+	0	4.44	0	0	0	0
5.42	67	0	+	0	4.48	0	0	0	0
5.58	72	0	+	0	4.54	0	0	0	0
5.88	78	0	+	0	4.60	0	0	0	0
6.10	80	0	+	+	4.85	0	0	0	0
6.30	95	32	+	+	5.20	4	0	+	0
6.50	103	82	+	+	5.80	61	0	+	0
6.72	101	100	+	+	6.18	66	0	+	+
6.88	111	103	+	+	6.42	73	32	+	+
7.09	106	99	+	+	6.70	96	47	+	+
7.30	92	75	+	+	6.85	89	75	+	+

INHIBITION UNDER STRICTLY AND SEMI-ANAEROBIC CONDITIONS

Experiment 7. Each tube in two sets of nutrient broth medium was inoculated with approximately 4.5×10^6 cells of culture 6 (*Flavobacterium* sp.), one set being incubated under strictly anaerobic conditions, and the other under the usual semi-anaerobic conditions. A similar experiment was made using culture 17 (*Achromobacter* sp.). The results (tables XI and XII) show that nitrite inhibited growth under both anaerobic and semi-anaerobic conditions, the only noticeable difference being that growth both in presence and absence of nitrite was never nearly as great under anaerobic as under semi-anaerobic conditions.

EFFECT OF SMALL INOCULA

Experiment 8. Each of the following four organisms was inoculated into a set of nutrient broth, individual tubes receiving approximately the number of bacteria given: (1) Culture 9 (*Achromobacter* sp.), 120 organisms per tube. (2) Culture 17 (*Achromobacter* sp.), 160 organisms per tube. (3) Culture 4a (*Pseudomonas* sp.), 550 organisms per tube. (4) *E. typhosa*, 190 organisms per tube. When the results of this experiment (table XIII) are compared with those given in tables VIII, IX and X for very much larger inocula of cultures 9, 17 and 4a, the following differences will be observed. In the case of cultures 9 and 17 nitrite inhibited growth completely at pH 5.88 or lower when large inocula were employed, whereas with the small inocula as used above growth was totally inhibited at pH 6.1 or lower. With culture 4a no difference was noticed when either large or small inocula were used. In the case of *E. typhosa* nitrite severely inhibited growth with the small inoculum employed.

TABLE XI. Growth of culture 6 (*Flavobacterium* sp.) in nutrient broth of different pH under anaerobic and semi-anaerobic conditions in the presence and absence of 0.02 per cent NaNO_2 (Inoculum 4.5×10^8 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Anaerobic growth				Semi-anaerobic growth			
	1 day		14 days		1 day		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
5.12	0	0	0	0	5	0	+	0
5.42	3.5	0	1.7	0	23	0	+	0
5.58	6.3	0	12.5	0	33	0	+	0
5.88	7.0	0	13.0	0	37	0	+	0
6.10	7.2	0	10.8	0	41	0	+	0
6.30	9.6	0	15.5	0	49	0	+	0
6.50	10.5	2	11.0	0	51	0	+	0
6.72	11.5	11.9	16.0	15.0	69	32	+	0
6.88	12.1	12.4	12.8	13.5	64	50	+	+
7.09	13.3	13.4	13.0	11.0	73	69	+	+
7.30	12.0	10.0	12.1	11.5	70	58	+	+

TABLE XII. Growth of culture 17 (*Achromobacter* sp.) in nutrient broth of different pH under anaerobic and semi-anaerobic conditions in the presence and absence of 0.02 per cent NaNO_2 . Inoculum 7.8×10^8 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Anaerobic growth				Semi-anaerobic growth			
	1 day		14 days		1 day		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
5.12	23	0	+	0	57	0	+	0
5.42	41	0	+	0	67	0	+	0
5.58	60	0	+	0	72	0	+	0
5.88	71	0	+	0	78	0	+	0
6.10	95	0	+	+	80	0	+	+
6.30	99	0	+	+	95	32	+	+
6.50	114	9	+	+	103	82	+	+
6.72	115	15	+	+	101	100	+	+
6.88	109	30	+	+	111	103	+	+
7.09	105	98	+	+	106	99	+	+
7.30	92	90	+	+	92	75	+	+

TABLE XIII. Growth of small inocula of cultures 9, 17, 4a and *E. typhosa* in nutrient broth of different pH with and without 0.02 per cent NaNO_2 . (Inocula: culture 9, 120; culture 17, 160; culture 4a, 550; and *E. typhosa*, 190 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Culture 9		Culture 17		Culture 4a		<i>E. typhosa</i>			
	14 days		14 days		14 days		1 day		14 days	
	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2	Control	0.02% NaNO_2
5.12	0	0	+	0	+	0	+	0	+	0
5.42	+	0	+	0	+	0	+	0	+	0
5.58	+	0	+	0	+	+	+	0	+	0
5.88	+	0	+	0	+	+	+	0	+	0
6.10	+	0	+	0	+	+	+	0	+	+
6.30	+	+	+	+	+	+	+	+	+	+
6.50	+	+	+	+	+	+	+	+	+	+
6.72	+	+	+	+	+	+	+	+	+	+
6.88	+	+	+	+	+	+	+	+	+	+
7.09	+	+	+	+	+	+	+	+	+	+
7.30	+	+	+	+	+	+	+	+	+	+

BACTERICIDAL ACTION

In the strict sense of the terms a bactericidal substance is one which renders an organism incapable of further growth, while a bacteriostatic substance hinders growth but does not render the organism incapable of further growth. It is obvious that it is possible for a given compound to be at the same time bactericidal and bacteriostatic, depending on the concentration employed, sensitivity of different bacterial cells, and various other conditions. The foregoing experiments have shown that sodium nitrite in 0.02 per cent concentration exerts a pronounced bacteriostatic action, and that it inhibits growth entirely in this concentration under certain conditions. The following experiments show that sodium nitrite is capable of pronounced bactericidal activity.

Experiment 9. Petri dishes containing Bacto nutrient agar 1.5 times its normal strength were inoculated with culture 9 and incubated for 2 days at 25°C . The resulting growth was suspended in a large volume of 0.01 M sterile phosphate buffer (pH 7.2) and centrifuged rapidly under aseptic conditions, thus concentrating the bacterial cells. The supernatant liquid was discarded and the organisms suspended uniformly in similar buffer solution. The suspension, which contained 203×10^8 organisms per ml. (viable count), was used immediately subsequent to preparation as follows.

Five ml. of bacterial suspension were placed in each of ten sterile, cotton-plugged, 50 ml., round-bottom centrifuge tubes. Each of six of these tubes received 5 ml. of 1 molar KH_2PO_4 solution (pH 4.45), the remainder receiving 5 ml. of 1 molar phosphate buffer (pH 7.3). To certain of the tubes 5 ml. of 3 per cent sterile NaNO_2 were added, water being added to the others (see table

XIV). After mixing the contents of the tubes gently they were incubated at 22°C., examinations being made at intervals. In this way the organisms were exposed to 1 per cent NaNO_2 for varying periods of time under both acid and faintly alkaline conditions.

The approximate number of organisms surviving the treatment was determined as follows. The tubes were centrifuged rapidly for 15 minutes in order to concentrate most of the bacterial cells, the faintly opalescent supernatant liquid being discarded after determining its pH. The precipitated organisms were resuspended in 15 ml. of sterile 0.01 M phosphate buffer (pH 7.2), mixed thoroughly, and viable bacterial counts made. It will be noticed (table XIV) that the pH of the solutions in the acid range varied between pH 5.55 and 5.80, and in the alkaline range between pH 7.20 and 7.50. Two factors appear to contribute to this slight variation, namely a decrease in pH due to the addition of NaNO_2 , and an increase in alkali resulting from incubation of the organisms in the absence of nitrite. It will be seen that 1 per cent NaNO_2 caused a very rapid fall in the numbers of viable bacteria in suspensions maintained at acid pH value. On the other hand above pH 7 it exhibited only a very slight bactericidal action.

Experiment 10. This experiment was carried out similarly to the foregoing one except that the bactericidal activity of 10 per cent and 0.02 per cent NaNO_2 was tested. The suspension of cells of culture 9 used contained approximately 229×10^8 organisms per ml. (viable count). In the case of bacteria exposed to

TABLE XIV. Influence of pH on bactericidal action of sodium nitrite using culture 9 (*Achromobacter* sp.) as test organism.

NaNO_2 concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$9,300 \times 10^6$
0	24 hr.	5.80	$5,670 \times 10^6$
1	30 min.	5.60	$1,620 \times 10^6$
1	2 hr.	5.60	137×10^6
1	6 hr.	5.60	4.1×10^6
1	24 hr.	5.60	0
0	30 min.	7.40	$12,300 \times 10^6$
0	24 hr.	7.50	$9,200 \times 10^6$
1	30 min.	7.20	$6,500 \times 10^6$
1	24 hr.	7.20	$3,500 \times 10^6$

10 per cent NaNO_2 the suspension was centrifuged a second time after suspending in phosphate buffer before preparing the final suspension, in order to remove the rather large amount of nitrite present in the first suspension. Bactericidal action was studied only in acid medium since the foregoing experiment showed that NaNO_2 (1 per cent) merely exerted minor bactericidal activity above pH 7. In order to ascertain whether osmotic or other effects due to high salt concentration were causing inhibition parallel tests were conducted with 10 per cent NaCl .

TABLE XV. Bactericidal action of 0.02 per cent and 10 per cent NaNO_2 using culture 9 (*Achromobacter* sp.) as test organism.

NaNO_2 concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$6,000 \times 10^8$
0	24 hr.	5.75	$6,600 \times 10^8$
0.02	6 hr.	5.60	$4,700 \times 10^8$
0.02	24 hr.	5.60	$3,900 \times 10^8$
10	30 min.	5.40	3,270
10	1 hr.	5.40	0
10	6 hr.	5.40	0

The results (table XV) show that exposure of the bacterial cells to 10 per cent NaNO_2 caused a very rapid decrease in the number of viable bacterial cells. Ten per cent NaCl exerted a negligible germicidal activity in comparison. In 0.02 per cent concentration NaNO_2 caused only a very slight decrease in number of viable organisms.

Experiment 11. An experiment similar to the above was carried out with culture 4, a species of *Micrococcus*, the growth of which had not been inhibited appreciably by 0.02 per cent NaNO_2 in previous experiments (Tarr 1941). The suspension employed contained approximately 165×10^8 organisms per ml. (viable count). The results (table XVI) show that both 1 and 10 per cent NaNO_2 exerted a markedly destructive effect upon the cells of this culture.

Experiment 12. In view of the fact that Bittenbender, *et al.* (1940) had reported that *S. aureus* survived exposure to 38.8 per cent NaNO_2 for 10 minutes this organism was also studied. The technique employed was similar to that outlined in previous experiments, the suspension of *S. aureus* used having approximately 667×10^7 organisms per ml. (viable count). The results (table XVII) show that in the case of this organism 10 per cent NaNO_2 exerted a very marked bactericidal action.

TABLE XVI. Bactericidal action of 1 per cent and 10 per cent NaNO_2 using culture 4a (*Pseudomonas* sp.) as test organism.

NaNO_2 concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$3,500 \times 10^6$
0	24 hr.	5.75	$2,200 \times 10^6$
1	30 min.	5.55	$3,500 \times 10^6$
1	6 hr.	5.55	1.2×10^6
1	24 hr.	5.55	0
10	30 min.	5.35	3,570

TABLE XVII. Bactericidal action of 10 per cent NaNO_2 using *S. aureus* as test organism

NaNO_2 concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$1,990 \times 10^6$
0	6 hr.	5.55	$2,370 \times 10^6$
10	30 min.	5.40	28,700
10	6 hr.	5.40	0

DISCUSSION

The present experiments have shown that in acid medium 0.02 per cent of sodium nitrite inhibits growth of two species of obligate anaerobes of the genus *Clostridium*, and also of certain facultative anaerobes when cultivated under strictly anaerobic or the usual semi-anaerobic conditions. Previous investigators (Lewis and Moran 1928, Tanner and Evans 1934) found that growth of obligate sporogenous anaerobes was inhibited by sodium nitrite only when concentrations normally greatly in excess of 0.02 per cent were used. Since these workers did not state the pH of the media which they employed, their failure to record bacteriostatic activity with small concentrations of nitrite may well have been due to the fact that these nutrient substrates possessed a neutral or faintly alkaline reaction.

The fact that growth of these obligate sporogenous anaerobes is inhibited by nitrite is of interest. The theory has been advanced that inhibition of growth of such organisms during meat-curing processes is due to hydrogen peroxide, a substance which is extremely toxic for *Clostridia* even in extremely small amounts, and which may accumulate as a result of inhibition of catalase by the hydroxylamine formed as an intermediate during the bacterial reduction of nitrate to nitrite (Jensen and Hess 1941). While this effect may well account for some of the inhibition of growth of such organisms during curing, it would appear that, since meat curing is usually carried out in somewhat acid medium, nitrites may also retard multiplication of *Clostridia* under such conditions.

It was found that the pH range over which nitrite inhibited growth of facultative anaerobes varied considerably with the substrate employed. In general the range was greatest when nutrient broth was used, and considerably less when using fish digest broth and synthetic medium. The reason for this was not ascertained. The fact that nitrite inhibited bacterial growth in a simple ammonium lactate medium would make it seem highly unlikely that this compound effects inhibition by combining with and inactivating some essential growth factor which might be present in a more complicated medium, or by inhibiting proteolytic or other enzymes which are presumably not utilized in a synthetic medium. There is little doubt that nitrite acts directly on the bacterial cell, but whether its action is specific or general is not clear.

It was found that sodium nitrite in fairly high concentration (1 and 10 per cent) exerted marked bactericidal action in acid medium, but not above pH 7. Bittenbender *et al.* (1940) reported that *S. aureus* was not killed by exposure to

38.8 per cent sodium nitrite for 10 minutes at pH values between 3 and 8. Since these investigators recorded only positive or negative growth after such treatment, and not the proportion of organisms rendered incapable of further multiplication, it would seem probable that in their experiments some, but not all, of the bacteria were inactivated. The present work has shown that *S. aureus* organisms are readily inactivated by 10 per cent sodium nitrite, depending on the length of time they are exposed.

In 0.02 per cent concentration sodium nitrite exerted only a very trivial bactericidal action. It would appear that such factors as concentration, pH, length of exposure and sensitivity of bacterial cells all contribute in determining whether nitrite exerts a bacteriostatic or bactericidal action.

Nitrite inhibited growth of the obligate anaerobes to about the same extent in cultures which were exposed to air during the early growth stages as in those not so exposed. Cultures of anaerobes not exposed to air grew at lower pH values than those exposed to it, and this is probably explained by the fact that vegetative cells of *Clostridia* are readily killed by exposure to oxygen (Knight 1941).

In concluding it is of interest to note that Meiklejohn (1940) found that growth of certain species of denitrifying *Pseudomonas* isolated from soil was strongly inhibited by nitrite in neutral or faintly acid solution, but not in alkaline medium, and that the degree of inhibition depended on the concentration of nitrite used.

SUMMARY

Growth of the obligate anaerobes *Clostridium botulinum* and *C. sporogenes* in fish digest broth was inhibited by 0.02 per cent sodium nitrite at acid pH. When vegetative cells of these organisms were exposed to air during the maximum growth phase multiplication did not take place at such a low pH as in cultures not so exposed. The degree of inhibition by sodium nitrite was not appreciably affected by such treatment.

Growth of certain species of facultative anaerobes was inhibited over a much wider pH range in ordinary nutrient broth than in fish digest broth or synthetic medium.

In nutrient broth nitrite inhibited growth of certain facultative anaerobes when these were cultivated under either strictly anaerobic or semi-anaerobic conditions.

The size of inoculum employed exerted only a minor influence on the final inhibition of growth of certain facultative anaerobes by sodium nitrite in nutrient broth.

Growth of the human pathogens *Eberthella typhosa* and *Staphylococcus aureus* in nutrient broth was inhibited by sodium nitrite in acid medium.

Sodium nitrite can act in either a bactericidal or bacteriostatic capacity, depending on such conditions as concentration employed, pH, length of exposure and sensitivity of bacterial cells, but its mode of action has not as yet been ascertained.

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Diurnal Variations in Feeding Activity of Young Salmon and Trout

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ABSTRACT

Young salmon and trout ordinarily take little food from about 10 p.m. to about 5 a.m. This does not seem to be entirely due to changes in temperature or light conditions. It seems probable that the fish are habituated to sleep during the hours of darkness when less food is available. A slight depression in feeding also occurs at mid-day due to rising temperature and, possibly, strong light. Extremely high temperatures at first depress, then bring about a complete cessation of feeding. After exposure to high temperatures salmon and trout will not take food again for some time, even if they find themselves in rather cool water.

Daily variations in the feeding activity of young salmon (*Salmo salar* L.) and trout (*Salvelinus fontinalis* Mitchill) have been investigated in the course of a general inquiry into the factors responsible for good or bad salmon and trout angling. The experiments were done at Moser River, N.S., during the summers of 1940 and 1941, as a part of the Atlantic Salmon Investigation being carried on under the direction of A. G. Huntsman. The writer is indebted to Dr. Huntsman for numerous suggestions.

Preliminary work showed that the feeding activity of both salmon and trout did vary with the time of the day. In particular, two physical factors vary during the 24 hours of each day, viz., temperature and light. Either or both of these might be responsible for such daily variations in feeding. The experiments, therefore, fall logically into three main groups: *a*,—feeding in relation to the time of the day; *b*,—feeding controlled in relation to temperature and light; and *c*,—feeding controlled in relation to light intensity and temperature.

METHODS

The fish were kept in wooden boxes approximately 60 cm. long, 45 cm. wide, and 15 cm. deep. The ends and top of the boxes were of wire screening. The bottom was covered with oilcloth to prevent injury to the fish and to facilitate removing uneaten food during the course of the experiments. The boxes were kept either in Moser river or Holman brook. This permitted a contrast in temperature, since the brook is usually from 4 to 6°C. cooler than the river during the heat of the summer. The light entering the boxes was controlled somewhat

by covering the boxes with alder branches or several thicknesses of jute cloth. For feeding fish in total darkness a light-proof box was constructed (figure 1).

In all experiments, precautions were taken against a conditioning of the fish to feed in relation to the activities of the person feeding them. McKenzie (1935) has shown how readily cod will become so conditioned. In these experiments we avoided disturbing the fish at the time of feeding, and took special care to introduce the food in a different fashion each time.

Repeated experiments showed that for salmon smolt, trout smolt or sea-run trout either *Gammarus* sp. or earthworms were very satisfactory food. For these groups of fish gammarids were most frequently used since they are abundant,

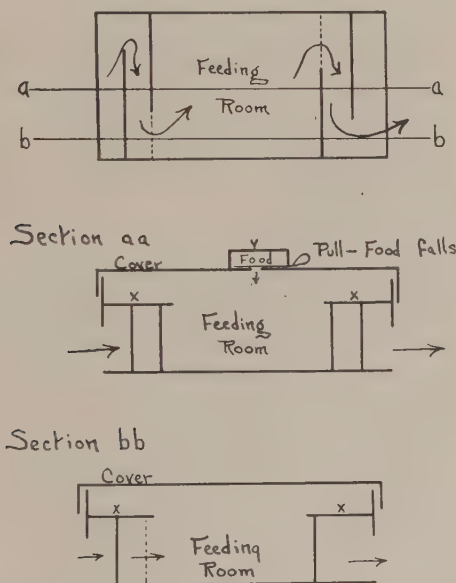


FIGURE 1. Light-proof feeding box (scale—1.3 cm. to 30.0 cm.). Upper,—as seen from above, with end-shelves ("x" in middle and lower figures) omitted; middle and lower,—sections through the box at levels "aa" and "bb" respectively. Heavy lines are black-painted boards; broken lines,—wire screening; arrows,—direction of water flow; and "y" a small light-proof box over a metal shutter which pulls out to drop food into the box.

fairly uniform in size, will swim about, and seem to be very attractive to the fish. Salmon parr and smaller ("brook") trout would not take the gammarids consistently. They preferred earthworms when cut into pieces 2 to 3 cm. long.

In the experiments precautions were taken to insure that the fish were always hungry. The amount of food which any group of fish could consume in a day was determined. In the experiments the fish were fed something less than this full amount. In this way it was hoped to keep the physiological factor of hunger relatively constant, and to investigate the physical factors of light and temperature. Feeding activity was judged by the percent of gammarids or earthworm-pieces eaten during a certain period of time. In any particular

experiment a specific amount of food was introduced into the box and, after a definite period, the remains noted.

The number of fish studied was not large (17 salmon and 9 trout). However, the different experiments were repeated often enough to establish the order with reasonable certainty. In all, 147 feeding experiments (72 on salmon and 75 on trout) were performed.

TIME OF DAY

EXPERIMENTAL

The graphs in figure 2 are typical for the daily feeding activity of salmon parr. In these experiments five 3-year-old parr were given 3 pieces of earthworm to eat during one hour. Preliminary trials had shown this amount of food to be about half that which they would eat in that time when without food for at

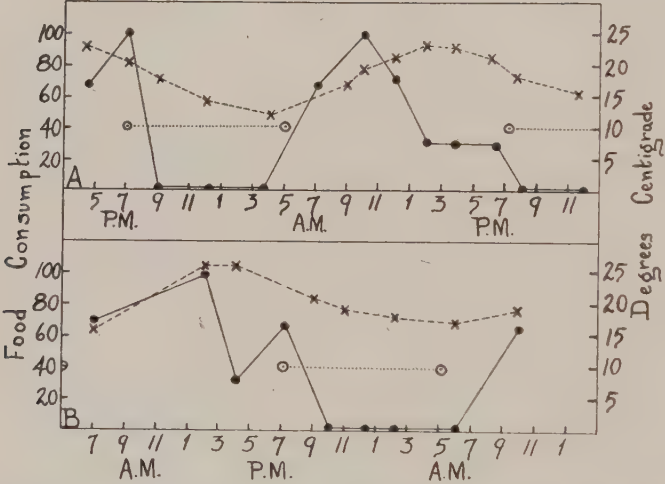


FIGURE 2. Salmon parr feeding in relation to time of day. Continuous line,—food consumption; broken line,—temperature; dotted line,—period from sunset to sunrise; A,—for August 14 and 15; B,—for August 19 and 20.

least 2 hours. It is evident, from the graph, that the fish cease feeding in the evening when it gets dark, usually by 9 p.m., although once they apparently took a small amount of food at 11 p.m. They do not commence feeding again until 5 a.m. or later when it is getting quite light. The feeding activity increases in intensity throughout the morning, but often slackens off during mid-day. Parr usually have another period of active feeding in the evening (figures 2 and 5).

Figure 3C shows the feeding activity of a group of 3 salmon smolt when given 12 gammarids for a 15-minute period. Figure 4A and B shows the activity of two trout smolt given 6 gammarids for a 15-minute period. Preliminary trials showed that 1 salmon smolt would take as many as 8 gammarids 5 times per day, and that 1 trout smolt would take at least one-half that number. The figures suggest that, although smolt (salmon or trout) may take some food at any time during the day or night, the trend in activity is the same as that of the salmon

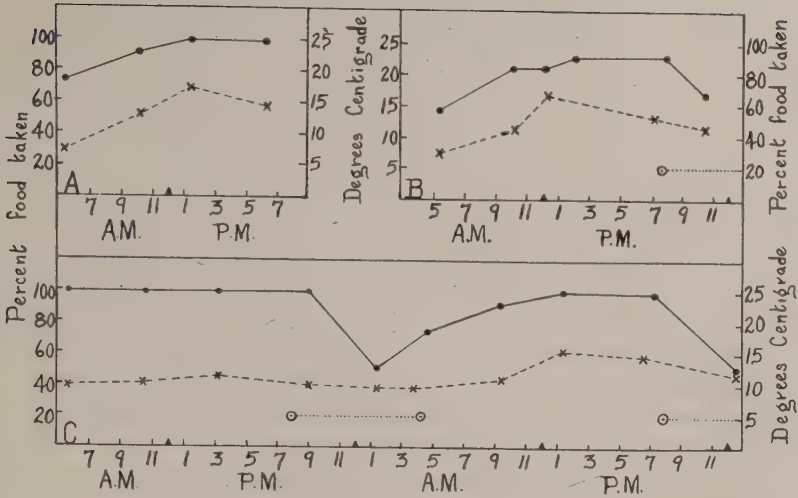


FIGURE 3. Salmon smolt feeding in relation to time of day. Continuous line,—food consumption; broken line,—temperature; dotted line,—period from sunset to sunrise; A,—for June 13; B,—for June 14; and C,—for June 16 and 17.

parr. Feeding is definitely depressed during the hours of darkness. Further, the fish feed with a lower intensity of light during the evening than during the early morning hours. Except for this nocturnal depression, the salmon smolt under observation (fully transformed fish after mid-June) were found to feed avidly at all times (fig. 3). The trout smolt, however, (fig. 4) show a mid-day depression similar to that mentioned above for salmon parr. For salmon smolt, and to a lesser degree for trout smolt, the food requirements are very great and it is

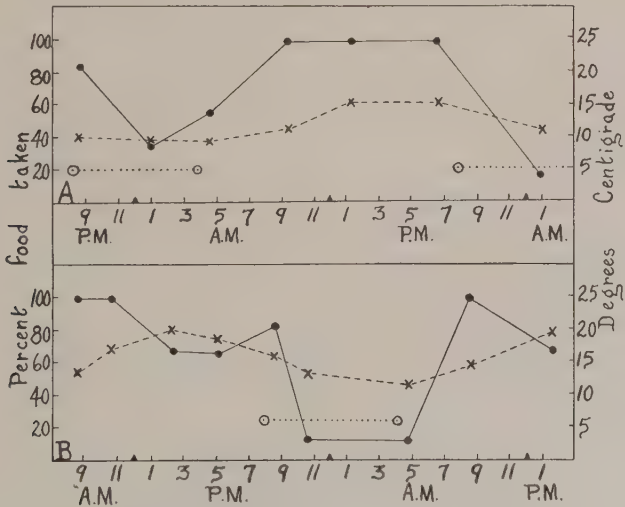


FIGURE 4. Trout smolt feeding in relation to time of day. Continuous line,—food consumption; broken line,—temperature; dotted line,—period from sunset to sunrise; A,—for June 16 and 17; B,—for June 19 and 20.

difficult to satisfy or overfeed the fish. Even with the greatest of care they rapidly become conditioned to the movements in the neighborhood of the boxes and take food avidly at all times.

DISCUSSION

Thus for both young salmon (parr and smolt) and trout (smolt) there is a definite nocturnal depression in feeding activity. Fish feed until after it is quite dark in the evening, but do not take much food again until 2 hours or more after day-break. They feed with a lower light intensity in the evening than during the early morning hours. It is as though the fish gradually resumed activity after a night of quiet. Further, there is a somewhat smaller and less consistent mid-day depression in the amount of food taken, and it would seem that the factors responsible here are of less magnitude. If the fish requires much food and is very hungry (as in smolt) the depression is not evident. On the other hand, fish with a lower rate of metabolism (parr) respond to this depressing factor, whatever it may be.

TEMPERATURE

LOWER RANGES

EXPERIMENTAL

The daily feeding curves (figs. 2, 3 and 4) are plotted in relation to the temperature. An examination of these figures suggests that temperature is not responsible for the nightly depression in feeding activity, but that it is probably responsible for the mid-day depression. In all cases the evening depression coincides with falling temperatures. This is not in line with our other experiments (figs. 6 and 7) which suggest that high, not low, temperatures depress feeding. It has, however, been shown (Allen 1940; Blair unpub.) that very low temperatures, below 6 or 7°C., do depress the feeding activities of young salmon. However, the evening low temperatures of our experiments are within the range of what Blair found to be optimum for feeding. It seems pertinent, also, that the nightly feeding depression was observed with an almost constant temperature during the whole of the 24-hour period (fig. 3C); and that, in this same experiment, the fish took food all day with temperatures as low as those coinciding with the nightly depression evident in the experiment of the previous day (fig. 3B). Temperature, then, in itself does not offer a satisfactory explanation for the nocturnal depression in feeding.

Figures 2, 3 and 4 indicate that the mid-day depression in feeding is largely a temperature effect. This is particularly well shown in the feeding curves for the trout smolt (fig. 4). In this series a mid-day depression occurred with a maximum temperature of 20°C., but was not evident with a lower mid-day temperature of 14° (fig. 4A and B). However, to make a proper study of the mid-day feeding depression it was found necessary to turn to experiments on salmon parr; for, as has previously been stated, salmon and trout smolt feed most avidly and do not show a mid-day depression with the same degree of regularity unless water temperatures approach lethal levels.

In the experiments on salmon parr, four 2-year-old fish were given 6 pieces of earthworm for a 15-minute period. The boxes were kept covered with 4 thicknesses of jute cloth, so that the fish were always in light of rather low intensity and any changes in feeding activity could not be attributed to strong light.

A long series of experiments was carried out during June and July 1941 (fig. 5). In Holman brook, between June 18 and June 24, maximum mid-day temperatures of 20°C. were attained and feeding was depressed in direct relation to such rises (fig. 5A for June 19). On June 24 the parr were transferred to the Moser river, where the temperatures were regularly 4 to 6° higher than in the brook. The feeding activity was of the same general nature as in Holman brook. On July 3 (fig. 5B), near the beginning of a period of rather high temperature

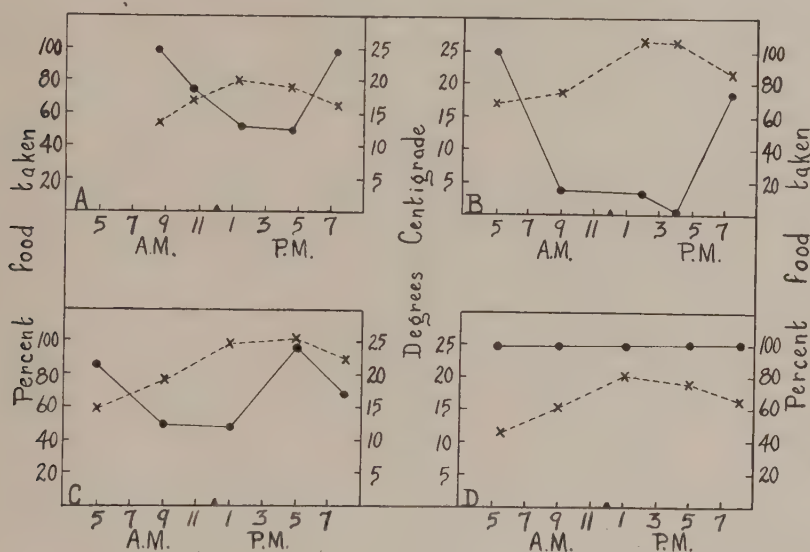


FIGURE 5. Salmon parr feeding in relation to temperature. Continuous line,—food consumption; broken line,—temperature; A,—for June 19; B,—for July 3; C,—for July 5; and D,—for July 12.

(maximum mid-day temperature on this date was 27.0°), feeding practically ceased. When the fish were somewhat acclimatized to the warm water the depression in feeding was less (fig. 5C for July 5). Further, the data indicate that, with prolonged high temperatures, feeding seems to become somewhat irregular and erratic. For example, more food was taken at 5 p.m. than at 7.30 p.m. on this particular day; and this is contrary to expectations. On July 11 the fish were moved back to the cooler waters of Holman brook. No mid-day depression in feeding was then found with temperatures going to 20° (fig. 5D). Evidently the fish became so acclimatized to high temperatures while in the river that a mid-day temperature of 20° was not high enough to cause a slackening in feeding activity such as occurred in June (fig. 5A), when the fish were acclimatized to somewhat lower temperatures.

The trout smolt feeding experiments confirm the above findings for parr. Figure 4 shows no depression in feeding with a noon temperature of 15°. There was, however, a definite slackening in feeding activity with a noon temperature of 19°.

DISCUSSION

Exposures to temperatures below the normal or customary range have been found to depress feeding in salmon (Allen 1940; and Blair unpub.). A similar temperature relationship has been demonstrated for herring (Battle *et al.* 1936; Johnson 1939), plaice (Dawes 1930), bass, etc. (Hathaway 1927; Markus 1932), and cod (McKenzie 1934 and 1935). On the other hand, abnormally high temperatures likewise depress feeding activities (Blair unpub.; Langford 1938; McKenzie 1934, 1935; and Pentelow 1939). The consensus among these workers is that there is an optimum temperature for feeding, and that either too high or too low a temperature will have a depressing effect.

However, as far as the writer can ascertain, no one has attempted to analyze diurnal variations in feeding activity. In the experiments of the above mentioned workers, fish were either acclimatized to some particular temperature and then feeding activity assessed (Hathaway 1927; Johnson 1939) or the long-range seasonal changes analyzed in relation to food consumption (Langford 1938; McKenzie 1934 and 1935). It may not be surprising in the light of this work that salmon and trout do show a mid-day depression in feeding activity. However, it seems worth recording that not only long range changes in temperature are effective, but that even within the daily temperature variations feeding will fluctuate. Our data show further that the precise point at which such slackening in feeding will occur depends on the previous acclimatization of the fish with respect to temperature.

APPROACHING THE LETHAL

EXPERIMENTAL

The feeding behaviour of trout exposed to continuous high temperatures seems to be rather pertinent to a consideration of the possible effects of high temperature on angling. In this work, the most consistent results were obtained with sea-trout. These fish, just returning from the sea, had been at lower temperatures than the other fish under investigation and proved to be more responsive to high temperatures.

In a series of experiments (fig. 6) two sea-trout (25 to 30 cm. in length) were given 2 gammarids for an hour period. It was found that these two fish would take at least 12 gammarids hourly during the day. Thus they were always hungry and fed regularly and satisfactorily. No attempt was made to prevent conditioning here, and the fish soon fed readily either day or night, provided the temperature did not go above 24°C. Darkness seemed to have little effect. Night feeding was carried out on three occasions, once on a dark night and twice in moonlight. On one of the latter nights the box was covered with canvas to keep out light. The food was always taken promptly. As we expect, however, temperature does have a profound effect. Between 24 and 25° feeding becomes

sporadic; that is, the food may or may not be taken. Above 25° the fish never took any food.

But the relationship of high temperature to feeding is much more complex than this. When exposed for 1 hour to temperatures rising from 25.0 to 27.2°C . the trout did not feed when placed in brook water at a temperature (22.5°) at which they normally take food (fig. 6B). In this experiment (fig. 6B) the temperature of the brook to which they were transferred then rose from 22.5 to 24.0° within the next $1\frac{1}{2}$ hours, and remained between 24.0 and 24.5° for a period of 3 hours. The temperature then fell and the fish took food for the first time between temperatures of 23.5 and 22.5° . Thus, apparent recovery from a short (1 hour) exposure to high temperatures occurred in 6 hours at most.

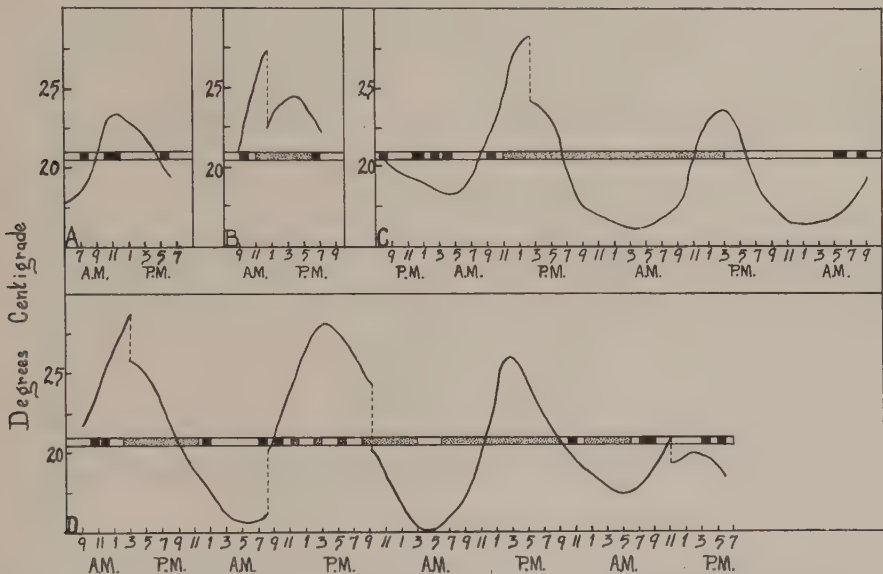


FIGURE 6. Feeding of sea-trout in relation to temperature and time of day. Continuous line,—temperature; interrupted line,—transfers from river to brook; broad band,—feeding activity. Food, placed in the box at the beginning of the time represented by a blocked area of the band, was eaten at the end of the period if the area is black, and was not eaten if the area is stippled.

When exposed for $3\frac{1}{2}$ hours to temperatures rising from 25.0 to 28.2°C ., and then transferred to water whose temperature was falling from 24° , the trout did not recover within 23 hours, which brought the experiment into the heat of the next day (fig. 6C). Beyond this point it was impossible to follow the results of this experiment in detail. However, on the second day the temperature did not rise higher than 23.5° , and the fish were feeding the following morning. Recovery took place somewhere between 23 and 39 hours. The important point is that a $3\frac{1}{2}$ hour exposure to temperatures higher than 25° may cause trout to suspend feeding for a period long enough to bring them into the heat of another day.

A similar effect was evident when the trout were exposed for 5 hours to temperatures ranging from 25.0 to 28.8°C. (fig. 6D), but feeding was resumed earlier (in about 7 hours) evidently because the temperatures for the 2 days prior to the experiment had not been as high as in the previous case (13.3 and 14.4° minimum daily temperatures as compared to 19.0°), and also because the temperature during the experiment was above 26° for only 2 hours as compared with 2½ hours in the previous case. The experiment (fig. 6D) was continued into the second day, the fish being exposed for 8 hours to temperatures above 25°. For 6½ of these hours the temperature ranged from 26 to 28°. After this treatment feeding was not resumed within 24 hours, although the night temperature went down to 15°. The third day of the experiment was cooler and involved a 2½ hour exposure to temperatures ranging from 25 to 26°. Six hours later the fish took one gammarid, and 9 hours after that (during the fourth day of the experiment with a maximum temperature of 21°) started feeding sporadically.

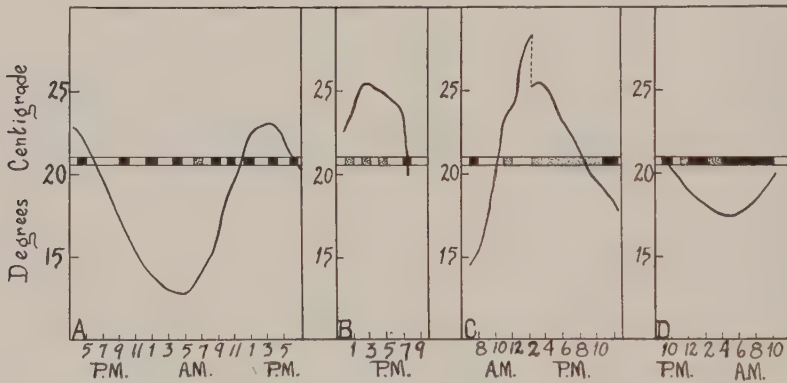


FIGURE 7. Feeding of "brook" trout in relation to temperature and time of day. Continuous line,—temperature; interrupted line,—transfers from river to brook; broad band,—feeding activity. Food, placed in the box at the beginning of the time represented by a blocked area of the band was eaten at the end of the period if that area is black, and was not eaten if the area is stippled.

A similar set of experiments was carried out with 2 trout parr ("brook" trout). These fish were given 2 pieces of earthworm for an hour period. The data (fig. 7) confirm the results of the sea-trout experiments.

Attempts to perform similar experiments with young salmon were unsuccessful, because of their higher temperature relationship. Whereas the trout under observation ceased feeding at approximately 24°, the fresh water salmon did not cease feeding until temperatures of 27 to 28° were attained. In the Moser river such high temperatures are not sufficiently prolonged to do more than bring about an erratic feeding.

DISCUSSION

It seems evident that prolonged exposures to high temperatures, such as may be found in the Moser river during the summer, bring about a 24-hour or

longer cessation in the feeding of trout. This takes place in spite of the fact that the temperature may fall very low during the night—far below the point at which feeding is normally depressed. Hence, a series of hot days may cause feeding to become irregular even on succeeding cool days. So far as known, no one has pointed out this particular relationship, nor determined the time required for feeding to be resumed when depressed by high mid-day temperatures.

LIGHT

It would be interesting to know to what extent the nocturnal depression in salmon and trout feeding is due to the darkness. Is it that fish cannot see to feed when it is dark, or are they habituated to sleep during the night when food is less readily available? The latter explanation would seem to be suggested, since the young salmon and trout do take food after it is quite dark in the evening, but do not take any until after it is quite light in the morning (figs. 2, 3, and 4). However, this observation does not prove that fish do not locate their food by light receptors, since, even on the darkest of nights, some light can be detected.

EXPERIMENTAL

In an attempt to elucidate this matter, a completely light-proof box was constructed (fig. 1). Food was introduced into the feeding room through a shutter which could be operated without disturbing the fish or letting in any light. The feeding reactions of both salmon and trout were tested in this box.

When salmon parr were in total darkness they took 100% of the food, but only 62.5% when the box was uncovered (table I). This was so in spite of the fact that the feeding reaction in the light-proof box was tested during the middle of the day when feeding might be expected to be somewhat less avid due to higher temperatures.

TABLE I. Feeding by 2 salmon parr in light-proof box (July 26, 1941). Fish were placed in box at 6.00 a.m. Cover was put on at 8.15 a.m., removed temporarily at 12.00 noon, and finally at 3.00 p.m.

Time of introducing food	Water temperature (°C.)	Previous light conditions	Per cent. of 4 earthworm-pieces eaten after 15 minutes.
8.00 a.m.	15.6	Full daylight for 2 hr.	50.0
11.45 a.m.	17.0	No light for 3½ hr.	100.0
2.45 p.m.	17.6	No light for 2¾ hr.	100.0
6.00 p.m.	17.5	Full daylight for 3 hr.	75.0

One trout smolt that had been held for 2 months and took food very readily, was fed in the light-proof box on two successive days. It took its complete

ration of 4 earthworm-pieces as promptly in darkness as in light. In every test it took all the food in 5 minutes. The reaction was tested after the fish had been in darkness for 2 hours, and after it had been in the dark for only 5 minutes. No differences in feeding activity were observed.

A series of four, day-long, feeding experiments were carried out in the light-proof box with 2 sea-run trout, which had been taking regularly 4 earthworm-pieces in a 15-minute period. In 12 different feedings the trout took the food as readily (exactly the same on the average) in the dark box as in the uncovered box. Moreover, it was shown for these fish also, that no preliminary adjustment to darkness was necessary before the food could be located. Different lengths of time, from 5 minutes to 2 hours, were tried. Also, the fish were found to take food as quickly, if not more so, in darkness as in light. The food was often completely gone when the box was opened 60 seconds after the introduction of the food. It took slightly longer when the box was uncovered, since the fish did not take the food readily until the worker had moved away.

DISCUSSION

From all these experiments it would most certainly seem that experimental salmon and trout can feed as readily in darkness as in light. Moreover, no prolonged period of adjustment is necessary before they can locate the food. And, further, it seems likely that salmon and trout may, if necessary, locate food by a receptor other than a light receptor. With regard to fish feeding in the absence of light, Battle *et al.* (1936) and Johnson (1939) found that herring were unable to feed in the dark, and required light of the intensity of moonlight to locate their food. This is certainly not true for young salmon and trout. They do not ordinarily feed in the night, but do feed readily in the absence of light during the day.

Although the literature contains few references to the feeding reactions of fish in complete darkness, there are a number of references to the effects of changes in illumination. Hathaway (1927) concluded that moderate changes in illumination produce no perceptible effects on food consumption; and further that, in three out of four cases, a little more food was taken in dim than in bright light. Certain of our results are suggestive of the same thing, viz., that strong light may depress feeding activity. Huntsman (1942) finds that salmon tend to avoid strong light by keeping in the deeper waters during the mid-day, while VanSomeren (1940) has observed that trout rise for insect larvae chiefly during the periods of low light intensity in the morning and evening. On the other hand, McKenzie (1935) concluded for cod that the changes in light intensity from early morning to dusk were without effect on the feeding. As far as our experiments are concerned the most suggestive data were obtained in the experimental feeding of fish in the light-proof box. In the limited number of observations on parr, the fish took, on the average, more food in darkness than in strong light. Also, for all groups of fish, feeding in darkness was frequently done at the time of day when temperatures were highest and light strongest, but the relative depression which often occurs at mid-day was not evident. These facts at least suggest that strong sunlight depresses the feeding activities of young salmon and trout.

SUMMARY

Both young salmon and trout show diurnal variations in their feeding activity. They always feed somewhat less avidly from 1 to 2 hours after sunset until 2 to 3 hours after sunrise. In addition, feeding activity is frequently depressed during the middle of the day.

The nightly depression in feeding is not entirely due either to temperature or to the absence of light. It occurs both with a falling temperature and with a steady 24-hour temperature. Also, salmon and trout are not entirely dependent on light receptors to locate their food, as shown by the fact that they take food readily if fed in darkness during the day time. Rather it seems that salmon and trout are habituated to sleep during the hours of darkness when food is less likely to be available, and that feeding is gradually resumed during the morning hours.

The slackening in feeding at mid-day is due primarily to rising temperature, although strong light is a possible associated factor.

High temperature first depresses feeding activity and then brings about a complete cessation of such activity. The precise temperature relationship depends on previous acclimatization (fish acclimatized to high temperature will feed at much higher temperatures), and on the type of fish (trout cease to feed at lower temperatures than do salmon).

If feeding is entirely suspended because of high temperature, it may not be resumed when the temperature again falls within the normal range. The fish must have sufficient time to recover at a lower temperature.

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